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Porins from Gram-Negative Bacteria in Lipid Bilayer Membranes

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The cell wall of gram-negative bacteria consists of three different layers, the inner membrane, the peptidoglycan layer, and the outer membrane (15,6). The inner membrane acts as a real permeability barrier and contains besides the respiration chain a large number of different transport systems for substrates. The outer membrane acts in contrast to this as a molecular filter with a defined exclusion limit for hydrophilic substances (16). These permeability properties are coupled to the presence of a class of major proteins in the outer bacterial membrane, called matrix proteins (17) or porins (11,12). The porins have been isolated from the outer membrane of Escherichia coli, Salmonella typhimurium and Pseudomonas aeruginosa (9,12,13). Experiments with reconstituted vesicles in the presence of porins from enteric bacteria have shown that the maximum molecular weight for permeable substances is in the case of E. coli and S. typhimurium between 600 and 700, whereas molecules with molecular weights up to 6000 can penetrate the reconstituted vesicles in the presence of porin F from Ps. aeruginosa (9,12,23).

Experiments with reconstituted vesicles provide excellent information about the presence and the size of the pores formed by a porin. More detailed information about the pore interior and the pore selectivity can be obtained from experiments with lipid bilayer membranes. In the following we shall give a short review on the properties of porins from E. coli, S. typhimurium and Ps. aeruginosa in lipid bilayer membranes. We shall show that the porins of these gram-negative bacteria form water filled pores with diameters between

1.2 and 2.2 nm and that most of the pores have a certain selectivity for ions (3-5). The conductance of the porin pores is in the order of 0.1 to 0.6 nS for 0.1 M alkali chloride solution.

Reconstitution of Porin into Lipid-Bilayer Membranes

The porins were isolated from the outer membrane of the different bacteria according to standard procedures using either dodecylsulfate (SDS, E. coli and S. typhimurium) or Triton X-100 (Ps. aeruginosa) as detergents (9,12,13). All proteins appeared to be homogeneous as examined by SDS-gel electrophoresis. The molecular weight of the single porin monomers was in the order of 40 000 Dalton whereas the conductive unit was in all cases a trimer. Dissociated monomers were found to be inactive in lipid bilayer membranes and reconstituted vesicles (14). The stock solutions contained 0.1% SDS or Triton X-100, 3 mM sodium azide, 5 mM Tris-HCl pH 8 and between 0.1 and 1 mg/ml protein. They were kept in the refrigerator or in the freezer and remained active under these conditions for at least 3 months.

The addition of porin from the stock solution to the aqueous phase bathing a lipid bilayer membrane led to a strong increase of the membrane conductance. A typical experiment of this type is given in Fig.1. Porin monomers from S. typhimurium strain SH 5551 (40 000 Dalton M.W.) were added in a final concentration of 100 ng/ml to a black membrane from egg-phosphatidylcholine/n-decane. After an initial lag of 4 min, presumably due to diffusion of the protein through unstirred layers, the conductance increased by about three orders of magnitude within about 30 min. Only a slight further increase (as compared with the initial one) occurred after that time. It has to be noted, however, that the membrane conductance increased continuously until membrane breakage in the presence of all porins. It is interesting to note that the detergents had only a small influence, if any, on porin reconstitution. This was shown by using different detergents and detergent free porin from osmotic shock solution (1,2).

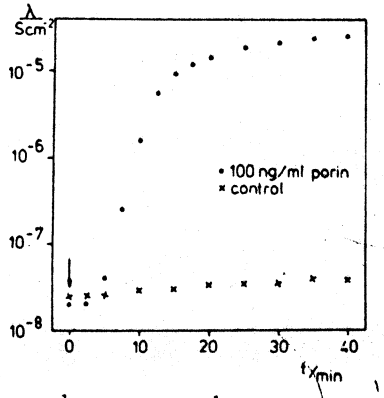


Fig.1 Specific membrane conductance as a function of time after addition of 100 ng/ml trimers of S. typhimurium strain SH 5551 (40 K) to a black membrane from egg-phosphatidylcholine/n-decane (arrow). The crosses represent a control experiment where only 10 µg/ml SDS was added to another membrane. MKCl, 25°C.

Since a steady conductance level could not be reached in the experiments with all porins, the dependence of the conductance on various parameters was difficult to obtain. A meaningful comparison was possible, however, on the basis of similar experiments as presented in Fig.1 and using the conductance value at a fixed time after addition of the protein. Fig.2 shows the influence of the membrane composition on the incorporation of the porins from S. typhimurium strain TA 1014 (38 K, 39 K and 40 K porins) into the membranes. With membranes made from oxidized cholesterol or of monoolein the conductance is about two to three orders of magnitude larger than for membranes made from phospholipids. A similar "lipid specificity" was also found for the porins from E. coli and Rs. aeruginosa (3,5).

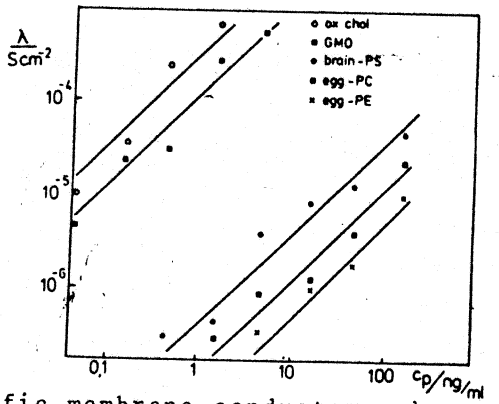


Fig.2 Specific membrane conductance λ as a function of the concentration of trimers from the Salmonella strain TA 1014 (38 K, 39 K, 40 K), in the aqueous phase; 1 MKCl; T=25°C. Less than 5 µg/ml SDS was present in the aqueous solutions.

Current-Voltage Curves

Fig.3 shows the current-voltage behavior of two membranes from oxidized cholesterol/n-decane in the

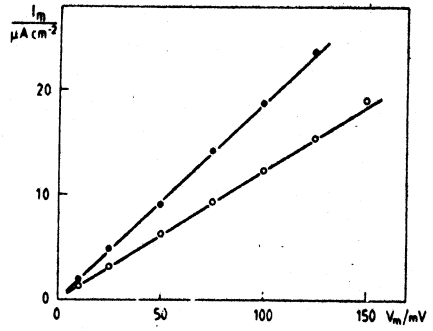


Fig.3 Current vs. voltage characteristics of two membranes from oxidized cholesterol/n-decane doped with porin F from Ps. aeruginosa. The aqueous phase contained 20 ng/ml porin, 0.5 μg/ml Triton X-100 and 1 M NaCl; T=25°C.

presence of porin F from Ps. aeruginosa. The current observed was a linear function of the applied voltage up to at least 150 mV. Even for an application of 100 mV for more than 30 s no current decrease but sometimes a slight increase was found presumably caused by the facilitated incorporation of the porin into the membranes by the high electrical field. Linear current voltage relationships were also found for the porins from E. coli and S. typhimurium (1,4). The results strongly suggested that no voltage is required to initiate the single conductance unit i.e. the pores were not voltage gated.

Single Channel Analysis

When the different porins were added in small concentrations (1-10 ng/ml) to the aqueous phase bathing a black membrane, the membrane current at a given voltage started to increase in a stepwise fashion. These current fluctuations were not observed when only the detergents SDS or Triton X-100 were added to the aqueous phase. Fig.4 shows experiments in the presence of porin from E. coli (trace 1) and of porin F from Ps. aeruginosa (trace 2). As can be seen from Fig.4 most steps were directed upwards whereas terminating steps were only rarely observed. This was also found for the different porins from S. typhimurium. The lifetime of all porin pores was at least one minute as derived from records extending over long times. Only

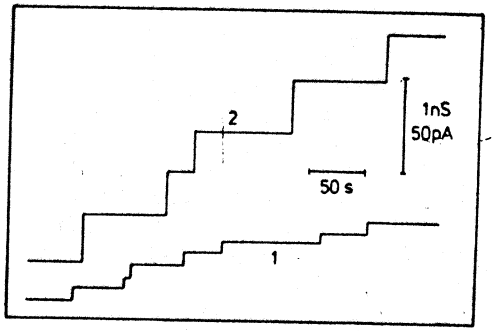


Fig.4 Stepwise increase of the membrane current in the presence of 0.1 ng/ml porin from E. coli (trace 1) or 5 ng/ml porin F from Ps. aeruginosa (trace 2) to the aqueous phase containing 0.1 M NaCl; T=25°C. The membranes were formed from egg-phosphatidylcholine/n-decane; V_m=50mV.

pores observed in the presence total outer membrane from Ps. aeruginosa (8) had a much shorter lifetime in the order of 50 to 100 ms, although the absolute level of the pore conductance was not changed as compared with isolated porin F (5).

Most of the conductance fluctuations obtained with the different porins were not uniform in size but distributed over a certain range. Histograms of the conductance steps measured with porin F from Ps. aeruginosa and porin from E. coli are shown in Fig.5 A and B. The fluctuations of the E. coli porin varied about

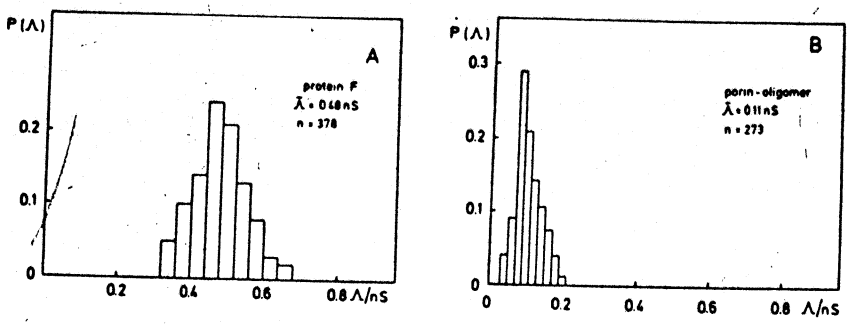
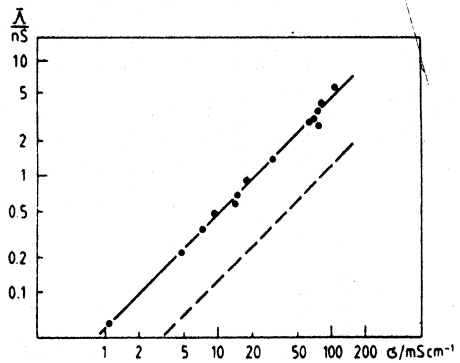


Fig.5 Histogram of the conductance fluctuations observed with membrane from egg-phosphatidylcholine/n-decane in the presence of porin F from Ps. aeruginosa (A) or of porin from E. coli (B), 0.1 M NaCl; T=25°C. n is the number of single steps; V_m=50mV.

our fold, whereas a factor of about two was between
ie smallest and the largest conductance steps of po-
in F. Similar distributions were found for all porins
ily the recently discovered anion channel formed by
orin P from Ps. aeruginosa gave a sharp distribution
f the conductance fluctuations (10).

Single channel measurement in the presence of the
orin were performed with a variety of different salts
id concentrations. From similar records to those
iven in Fig.4 the average conductance increment $\bar{\Lambda}$ was
btained by measuring a sufficient number (at least
0) of individual events. For all porins described in
his short review (except porin P from Ps. aerugi-
osa (10) the average pore conductance $\bar{\Lambda}$ was
linear function of the specific conductance σ of the
queous phase, i.e. the ratio $\bar{\Lambda}/\sigma$ varied only litt e
n contrast to variations of $\bar{\Lambda}$ by two orders of mag-
tude. This is also reflected in Fig.6 where the
verage pore conductance $\bar{\Lambda}$ for the F-porin of Ps.
eruginosa is given as a function of σ. The data points



g.6 Average pore conductance $\bar{\Lambda}$ of protein F from Ps. aeruginosa given as a function of the specific conductance of the corresponding aqueous salt solution; T=25°C. The broken line shows the results obtained with E. coli or Salmonella porins (3,4).

uld be fitted with a straight line. The same is va-
d for the porin from S. typhimurium (38 K, 39 K and
) K) and from E. coli (broken line in Fig.6), although
ie ratio $\bar{\Lambda}/\sigma$ varied up to a factor of 4 for these po-
ns. This can be explained by the larger diameter of
ie Ps. aeruginosa pore which is also consistent with
ie larger value of $\bar{\Lambda}/\sigma$ (Fig.6) and the vesicle per-
eability assa sing carbohydrates of different sizes

(8,9). It is interesting to note that even large ions such as Hepes$^-$ and $N(CH_2CH_3)_4^+$ were able to pass through the porin pores with little or no interaction with the pore interior.

Ionic Selectivity of the Porin Pores

Further information on the structure of the conductance pathway created by the different porins may be obtained by studying the ionic selectivity of the pores by zero-current potential measurements. Fig.7 shows the results of such experiments obtained for E. coli porin oligomers and membranes from oxidized cholesterol/n-decane. The zero-current potential was found to be positive on the more dilute side of the membrane. This indicated that porin oligomers from

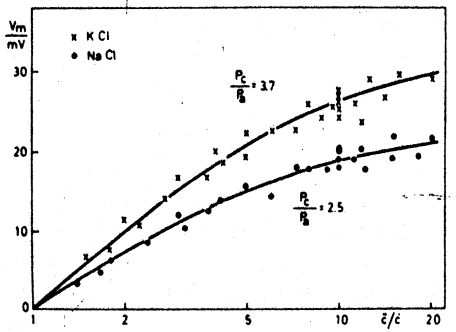


Fig.7 Zero current potentials V_m as a function of the ratio of the salt concentrations on both sides of membranes containing porin from E. coli. The membranes were made from oxidized cholesterol/n-decane; T=25°. V_m was positive on the more dilute side of the membrane, $c'=10^{-2}$M. The lines were drawn according to the Goldman-Hodgkin-Katz-equation with the specified values of the permeability ratio P_c/P_a (3).

E. coli form cation selective pores in lipid bilayer membranes. From the measured V_m and the concentration gradient c"/c' across the membrane, the ratio P_c/P_a of the permeabilities P_c for cations and P_a for anions was calculated according to the Goldman-Hodgkin-Katz equation (3). Table I shows the permeability ratios P_c/P_a for the different porins and KCl as a salt in the aqueous phase. The observed slight selectivity for cations or anions may be explained by the presence of negative or positive charges, respectively, in or near the pores. The anion selectivity of the P-porin of Ps. aeruginosa is presumably caused by a selectivity filter (10).

ble I Zero-c ent potentials V_m for different porins in the presence of a 10-fold KCl 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 from egg-phosphatidylcholine/n-decane; T=25°C. The ratio of the permeability P_c (cation) and P_a(anion) were calculated from the Goldman-Hodgkin-Katz equation.

Porin	V_m/mV	P_c/P_a
E. coli		
Ia/Ib(pH6)	30±3	4.6 ± 0.9
Ia/Ib(pH3)	-5±2	0.80± 0.1
Ia/Ib(pH9)	37±4	7.2 ± 2.2
Ic (pH6)	-28±3	0.24± 0.05
S. typhimurium		
40 K(pH6)	24±2	3.2± 0.5
Ps. aeruginosa		
F (pH6)	20±4	2.4± 0.4
P (pH6	-57±4	10^{-2}- 10^{-3}

Discussion

The experiments described in the short review show at porins isolated from the outer membrane of E. co-, S. typhimurium and Ps. aeruginosa are able to rm large ion-permeable pores in lipid bilayer membranes. A relatively easy method was used for the reastitution experiments. The protein was simply added the aqueous phase and was incorporated by itself to the membranes. The insertion into the membranes presumably controlled by hydrophobic forces beise the reconstitution rate showed no dependence on e ionic strength in the aqueous phase (1,4). The reastitution rate was found to be strongly dependent the type of lipid used for membrane formation. The nductance and the ionic selectivity of the single nductance unit, however, was independent from the oid. We believe therefore that the reconstitution e does not reflect a specific lipid-protein interion. It seems moreover, that the insertion of the mers in the membranes is governed by a kinetic process, for tance the replacement of 100 lipid molecule (area nm^2) by one porin trimer (area 50 nm^2 (16)) which need a considerable energy (4).

The experiments presented here support the assumpn of large water-filled pores formed by the porins

of the different gram-negative bacteria. The large organic ions $Tris^+$, $N(C_2H_5)_4^+$ and $Hepes^-$ can penetrate the pores without detectable interaction with the pore interior. Furthermore, the pore conductance showed no saturation with increasing salt concentration in the aqueous phase and the single channel conductance of all salt solutions. The current voltage characteristic was ohmic which is also expected for a wide unselective channel. Nevertheless there exist some differences in the single channel conductances. The single channel conductance of the porin F from Ps. aeruginosa was considerably higher than was observed for the porins from E. coli and S. typhimurium (Table II). This indicates that the diameter of the F-porin pore

Table II Comparison of the pores formed by porins of gram-negative bacteria. The diameter d was calculated from the pore conductance in 1 M KCl according to $\bar{\Lambda} = \sigma \cdot \pi r^2/l$ (using σ = 110 mScm^{-1} and l = 7.5 nm).

Pore	$\bar{\Lambda}$/nS	d/nm	Area/nm^2
E. coli			
Ia	2.3	1.4	1.6
Ib	2.2	1.4	1.5
Ic	1.7	1.2	1.2
S. typhimurium			
38 K	2.4	1.4	1.6
39 K	2.2	1.4	1.5
40 K	2.4	1.4	1.6
Ps. aeruginosa			
F	5.6	2.2	3.8

is larger than the diameter of the other porin pores. Assuming that the porin pores are filled with a solution of the same specific conductivity as the external solution and assuming a pore length of 7.5 nm (corresponding to the thickness of the outer membrane (6,16)) according to the equation $\bar{\Lambda} = \sigma\pi r^2/l$, the average pore diameter d (=2r) and the cross section can be calculated. Table II shows the diameter and the cross section for the porins from the different gram-negative bacteria calculated from the conductance of the pores in 1 M KCl (σ = 110 mScm^{-1}). The diameter od the F-porin pore is considerably larger than the diameter of the other pores. The values for the diameter given in Table II are consistent with the results of the reconstituted vesicles where hydrophilic

solutes of molecular weights up to 6 0 (E. coli) up to 700 (S. typhimurium) and up to 6000 (Ps. aeruginosa) were found to be permeable through the porin channels (9,12,13).

The outer bacterial membrane acts as a molecular filter for hydrophilic solutes (6,15). The results obtained on lipid bilayer membranes support this finding. In particular we found no gating of the porin pores and the pores had a long lifetime in the order of minutes. Only the F-porin pores from Ps. aeruginosa showed a much smaller lifetime (50-100 ms) if total outer membrane instead of purified porin was present in the experiments. This has presumably to do with the high natural antibiotic resistance of this organism as has been discussed elsewhere (5) but a voltage controlled gating process could also not be detected in this case. Furthermore, the vesicle permeability assay (5-8) leads to open pores without applied voltage, a fact which supports the results obtained from lipid bilayer membranes. Our findings are in sharp contrast to the results of two publications by Schindler and Rosenbusch (18,19) where porin pores from E. coli have been studied with folded lipid bilayer membranes formed from reconstituted vesicles. In these experiments no pores have been found to be open after membrane formation at zero voltage and large membrane potentials have been needed to switch on ($V_m \approx 100$ mV) and to switch off ($V_{in} \geq 150$ mV) the porin pores (18,19). The control of membrane permeability by voltage gated pores in the outer membrane of gram negative bacteria, as suggested by Schindler and Rosenbusch (18) are however difficult to understand. The channel density in the outer membrane of gram negative bacteria is about 10^{12} pores/cm^2 (6,16) and the time constant of the membrane is very small under normal conditions (10 ns at 0.1 M salt (4)). Any membrane potential will drop immediately to zero and the Donnan potentials, which have been reported to be about 20-30 mV across the outer membrane of S. typhimurium and E. coli (20), are by far too small to reach 100 mV. We believe therefore that other reasons than voltage gated pores must be responsible for the observed voltage effects (18). One reason could be that the porin molecule are located only in one monolayer and do not penetrate the folded membranes. The high electric field could facilitate the insertion of the proteins into both monolayers of the folded membrane and this process could open the pores. Another explanation would be that the folded membranes do not contain any porin after formation for unknown reasons. The reached vesicles, which contain porin,

could fuse with the membranes under the influence of the high electric field. The latter possibility would also explain the large conductance jumps observed by Schindler and Rosenbusch (18). Similar jumps have also been reported for the fusion of porin containing vesicles with planar lipid bilayer membranes (7).

It is still an open question wether one porin trimer contains three pores (16) or only one pore (6). Our results suggest that one trimer contains only one pore. The pore diameter as calculated from the conductance data would be simply too small in the case of three pores in a trimer to account for the permeability of large hydrophilic solutes through the porin pores. One pore per trimer is also supported by the finding that porin monomers have been found to be inactive in reconstitution experiments with vesicles (14).

The study of the porin channels from gram-negative bacteria offers an elegant way for the investigation of the structure-function relationship of pores. The porin trimers are usually very stable and allow chemical modifications without damage of the protein structure (21). In particular, it is possible to change the ionic selectivity of the porin channels by the chemical modification (R. Benz and T. Nakae, unpublished results). Further interest arises from the study of strong ion selective pore like the P-porin from Ps. aeruginosa, which contains a small selectivity filter for anions (10) and R. Benz, R.K. Poole and R.E.W. Hancock, unpublished results).

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References

1. Benz, R., Janko, L., Boos, W., Läuger, P. (1978): Biochim. Biophys. Acta, 511:305-319.

2. Benz, R., Boehler-Kohler, B.A., Dieterle, R., Boos, W. (1979): J. Bacteriol., 135:1080-1090.

3. Benz, R., Janko, K., Läuger, P. (1979): Biochim. Biophys. Acta, 551:238-247.

4. Benz, R., Ishii, K., Nakae, T. (1980): J. Membrane Biol., 56:19-29

5. Benz, R., Hancock, R.E.W. (1981): Biochim. Biophys. Acta, 646:298-308.

6. Di Rienzo, J.M., Nakamura, K., Inouye, M. (1978): (1978): Ann. Rev. Biochem., 47:481-532.

7. Finkelstein, A., Cohen, F.S., Akabas, M. (1981): Biophys. J., 33:105a.

8. Hancock, R.E.W., Nikaido, H. (1978): J. Bacteriol., 136:318-390.

9. Hancock, R.E.W., Decad, G.M., Nikaido, H. (1979): Biochim. Biophys. Acta, 554:323-331.

10. Hancock, R.E.W., Poole, R.K., Benz, R. (1982): J. Bacteriol., (in press)

11. Nakae, T. (1975): Biochem. Biophys. Res. Commun., 64:1224-1230.

12. Nakae, T. (1976): Biochem. Biophys. Res. Commun., 71:877-889.

13. Nakae, T., Ishii, J. (1978): J. Bacteriol., 133: 1412-1418.

14. Nakae, T., Ishii, J., Tokonuga, M. (1979): J. Biol. Chem., 254:1457-1461.

15. Nikaido, H. (1979): In: Bacterial Outer Membranes, edited by M. Inouye, pp. 361-407. Wiley-Interscience, New York.

16. Nikaido, H. (1979): Angew. Chem., Int. Ed. Engl., 18:337-349.

17. Rosenbusch, J.P. (1974): J. Biol. Chem., 249: 8019-8029.

18. Schindler, H., Rosenbusch, J.P. (1979): Proc. Nat. Acad. Sci., USA, 75:3751-3755.

19. Schindler, H., Rosenbusch, J.P. (1981): Proc. Nat. Acad. Sci., USA 78:2302-2306.

20. Stock, J.B., Rauch, B., Roseman, S. (1977): J. Biol. Chem., 252:7850-7861.

21. Tokunaga, H., Tokunaga, M., Nakae, T. (1981): J. Biol. Chem., 256:8024-8029.