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Properties of the porin of *Haemophilus influenzae* type b in planar lipid bilayer membranes

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The major outer membrane protein (40 kDa) of the bacterium *Haemophilus influenzae* type b is a porin which forms transmembrane permeability channels. It has an exclusion limit for oligosaccharides of about 1.4 kDa. When this protein was added to the aqueous phase which was bathing a planar lipid bilayer, it caused the conductance of the membrane to increase by several orders of magnitude. At low protein concentrations (2–10 pM), the conductance of the membrane increased in a stepwise fashion with an average single-channel conductance of 1.1 nS in 1 M KCl. Single-channel experiments were performed with a variety of different salts. The conductance of single channels was proportional to the specific conductance of the aqueous solution which was bathing the membrane. Current through the pores was proportional to the applied voltage, indicating that these pores are not voltage-controlled. The 40 kDa porin was very slightly cation-selective: the pores were about 1.6-times more permeable to potassium ions than to chloride ions. These properties of the 40 kDa porin are those of large water-filled channels and are characteristic of most bacterial porins. The single-channel conductance of the porin is, however, much smaller than might be expected from its exclusion limit. A model is proposed which could explain the differences in apparent pore size.

Introduction

The outer membrane of Gram-negative bacteria constitutes a selective barrier which prevents most hydrophobic molecules and large hydrophilic solutes from diffusing across the cell wall and into the periplasmic space [1,2]. Small hydrophilic solutes such as sugars and amino acids can cross this membrane through water-filled channels which are formed by outer membrane proteins termed porins [3].

Porins have been isolated from a variety of prokaryotic organisms. Three methods have been used to estimate the size of the pores formed by these porins. The efflux assay [4,5] measures the retention of selected solutes by reconstituted membrane vesicles and gives an estimate of an exclusion limit for these solutes. The liposome swelling assay [5,6] determines the relative rates of swelling of reconstituted liposomes which have been diluted into isotonic solutions of low-molecular-weight solutes. Using the Renkin equation [7], one may then calculate a pore size. Finally, pore diameters may be estimated from the conductance of single channels which are reconstituted into

Abbreviation: Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid.

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planar lipid membranes [1,8]. Only the porins from *Escherichia coli* [3,8,9] and protein F from *Pseudomonas aeruginosa* [10–12] have been tested in all three of these reconstitution systems. Good agreement was found between the estimates of pore size using all three methods. The porins of these bacteria therefore form water-filled channels with effective diameters between 1 and 2 nm.

We have demonstrated that the outer membrane of *Haemophilus influenzae* type b is highly permeable to β-lactam antibiotics when compared with *E. coli* or with *P. aeruginosa* [13]. This high degree of permeability to small hydrophilic solutes was attributed to large water-filled channels in the outer membrane of this bacterium [14]. An estimate of the molecular weight exclusion limit was made by reconstituting vesicles of lipopolysaccharide and phospholipids with whole outer membrane protein extracts. The exclusion limit was about 1.4 kDa for oligosaccharides. The porin of *H. influenzae* of 40 kDa was purified to apparent homogeneity and could be reconstituted into vesicles of lipopolysaccharide and phospholipids. A molecular weight exclusion limit for the isolated porin matched the estimate using outer membrane vesicles [14]. Furthermore, some clinical isolates of *H. influenzae* have recently been described which did not produce any detectable chloramphenicol acetyltransferase activity but which were resistant to chloramphenicol. Resistance was attributed to an increased permeability barrier of the outer membrane and was correlated with the loss of the 40 kDa protein [15].

We have investigated further the properties of the 40 kDa protein of *H. influenzae* using black lipid membranes. This paper demonstrates that the porin from this organism forms water-filled channels similar to those of most bacterial porins. However, the values obtained for single-channel conductance across the porin were significantly smaller than might be suggested by its molecular weight exclusion limit.

Materials and Methods

Bacterial strain and porin isolation

The 40 kDa protein of *H. influenzae* type b ATCC 9795 was purified as follows [14]: whole cells were suspended in a solution of 2% cetyltrimethylammonium bromide; this treatment solubilized most of the cellular proteins. After centrifugation, cellular proteins were discarded and the pellet was resuspended in water. The proteins contained in the pellet were then solubilized by the addition of $CaCl_2$ to a final concentration of 1 M. These solubilized proteins were separated from the insoluble residue by centrifugation, concentrated by ethanol precipitation, and fractionated by chromatography on DEAE-Sepharose as described previously. The preparation showed only one band, of M_r 40000, when analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis [14]. OmpF and NmpC porins of *E. coli* were generous gifts of Dr R.E.W. Hancock, University of British Columbia, Vancouver, British Columbia, Canada.

Lipid bilayer experiments

Two compartments of a Teflon chamber were connected by a small hole: 0.1 mm^2 for single-channel experiments and 1.0 mm^2 for macroscopic conductance experiments. Lipid bilayers were formed across the hole with a solution of either 2.5% glyceryl monooleate (Sigma Chemical Co., St. Louis, MO) dissolved in *n*-decane or 2.0% oxidized cholesterol dissolved in *n*-decane. The latter was prepared by boiling a 4% suspension of cholesterol (Sigma) in *n*-octane for 6 h with reflux and oxygen bubbling [16]. Bilayer formation was indicated by the membrane's turning optically black to incident light.

The purified porin was added to the aqueous phase either before membrane formation or after the membrane had turned black. Conductance across the membrane was measured by applying a fixed transmembrane potential. A pair of Ag/AgCl electrodes was inserted into symmetrical solutions on both sides of the membrane. For acetate, sulfate and Hepes, $1 \cdot 10^{-4}$ M chloride was always present. An operational amplifier (Analog Devices type AD 40K) was used in a current amplifier configuration with a $1 \cdot 10^9$ Ω feedback resistor. This equipment allowed the current to be monitored on a storage oscilloscope (Tektronix) and recorded on a strip chart recorder. The specific conductance of the various salt solutions was measured with a conductometer (Radiometer, Copenhagen, type CDM2e).

For measurements of zero-current membrane potential, the membrane was formed in a 10 mM salt solution. Sufficient porin was added so that the conductance of the membrane increased about 100-fold within 20 to 30 min. The voltage was turned off and the instrument was switched to the voltage amplifier mode to allow the measurement of membrane potential; this value served as the reference potential. The salt concentration on one side of the chamber was raised by adding and stirring small volumes of a 3 M solution of the same salt. The potential reached its final value within 5 to 10 min. The Ag/AgCl electrode potential is due to the difference in Cl^- activity and was calculated using the Nernst equation [17]. The electrode potential was subtracted from the measured potential and the result was the membrane potential.

Results

Macroscopic conductance measurements

The purified pore-forming protein of 40 kDa from *H. influenzae* type b was added at 1 ng/ml to the aqueous solution which was bathing a lipid bilayer. An increase in membrane conductance of several orders of magnitude was observed before breakage of the membrane (Fig. 1). The conductance increased more rapidly when membranes were formed with oxidized cholesterol; such membranes were generally more stable than glyceryl monooleate membranes in the presence of high concentrations of the 40 kDa protein. With either lipid a similar increase was observed whether the protein were added before membrane formation or after the membrane had turned black. In the absence of added protein, membranes formed with either glyceryl monooleate or with oxidized cholesterol were stable and their specific conductance did not increase significantly over a period of 30 min. When higher concentrations of protein were added, the specific conductance of both types of membrane increased more rapidly but the membranes broke after a shorter time. Glyceryl monooleate membranes were used for most single-channel experiments. Because of their greater stability oxidized cholesterol membranes were preferred for measurements of zero-current membrane potential.

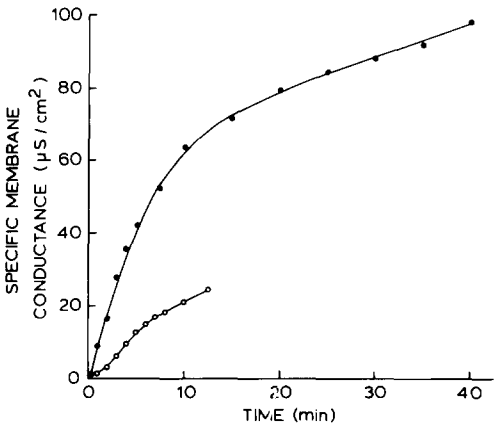

Fig. 1. Specific membrane conductance as a function of time after formation of the membrane. The aqueous phase contained 100 mM KCl and 1.0 ng/ml of the 40 kDa protein from *H. influenzae* type b. The membrane was made with 2.5% glyceryl monooleate (○) in *n*-decane or with 2% oxidized cholesterol (●) in *n*-decane over a hole of 1.0 mm^2 separating the two aqueous compartments. The voltage applied was 5 mV.

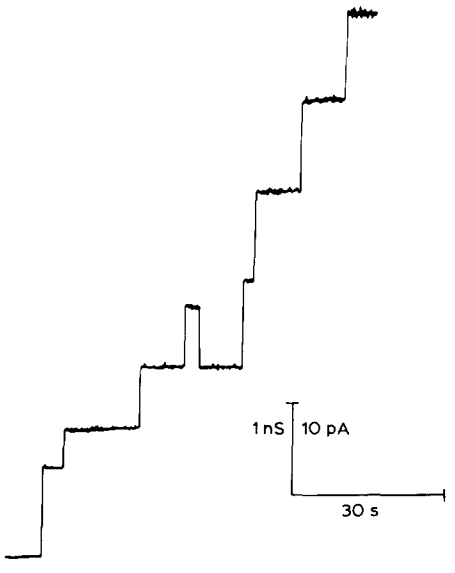

Fig. 2. Conductance steps caused by the addition of 0.3 ng of the 40 kDa protein per ml of the aqueous phase (1.0 M KCl) bathing a lipid bilayer membrane. The membrane was formed with 2.5% glyceryl monooleate in *n*-decane over a hole of 0.1 mm^2. The voltage applied was 10 mV. The record starts at the lower left. Most steps were directed upward, but occasionally a downward step was observed.

Single-channel experiments

When the 40 kDa protein was added to the aqueous phase in low concentrations (0.1 to 0.5 ng/ml), the conductance of the membrane increased in a stepwise fashion. A typical recording is shown in Fig. 2. The majority of conductance steps were directed upward, but downward steps accounted for 2–6% of the steps recorded during a given experiment. Only upward-directed steps were used in calculating the averages presented here even though conductance steps in both directions were of the same magnitude.

The distribution of single-channel increments of the 40 kDa protein in 1.0 M KCl and for a membrane of glyceryl monooleate is shown in Fig. 3. A similar distribution was obtained for a membrane of oxidized cholesterol. Although single-channel increments of about 1 nS were most frequent in 1.0 M KCl, the values ranged from 0.1 to 5.0 nS. Steps larger than 2.7 nS accounted for only 3% of the total number of steps recorded and could possibly be due to the simultaneous insertion of more than one pore into the membrane. The average conductance for single channels produced by the 40 kDa protein in 1.0 M KCl was 1.1 nS. As this value was much smaller than might be expected from the molecular weight exclusion limit of this porin [14], single-channel conductances were measured under the same conditions for OmpF and NmpC porins of *E. coli* (Table I). The

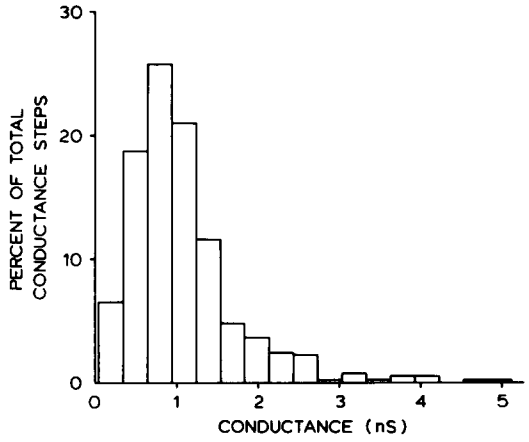

Fig. 3. Histogram of conductance steps in 1.0 M KCl. The membrane was made with 2.5% glyceryl monooleate in *n*-decane over a hole of 0.1 mm^2 separating the two aqueous compartments. The 40 kDa protein was added to a final concentration of 0.3 ng/ml and the voltage applied was 20 mV. The total number of steps examined was 353. The average single-channel conductance was 1.1 nS.

TABLE I

SUMMARY OF THE SINGLE-CHANNEL CONDUCTANCE AND ZERO-CURRENT MEMBRANE POTENTIAL FOR THE 40 kDa PROTEIN OF *H. INFLUENZAE* AND FOR TWO PORINS OF *E. COLI*

For single channel conductance experiments, the voltage applied was 20 mV. Zero-current membrane potential was measured in the presence of a 10-fold concentration gradient of KCl. The potential is that of the dilute side (10 mM) minus that of the concentrated side (100 mM). The ratio of P_c (permeability of the cation) to P_a (permeability of the anion) was calculated using the Goldman-Hodgkin-Katz equation.

Porin	Single-channel conductance in 1 M KCl [a] (nS)	Number of steps recorded	Zero-current membrane potential [b] (mV)	P_c/P_a
H. influenzae				
40 kDa	1.1	353	9.6 ± 1.6 (5)	1.6
E. coli				
OmpF	2.0	269	28 ± 4 (3)	4.2
NmpC	1.3	124	−26 ± 3 (3)	0.27

[a] The membrane was formed with 2.5% glyceryl monooleate over a hole of 0.1 mm^2.

[b] The membrane was made with 2% oxidized cholesterol over a hole of 1.0 mm^2. Data represent mean ± S.D.; the number of experiments is shown in parentheses.

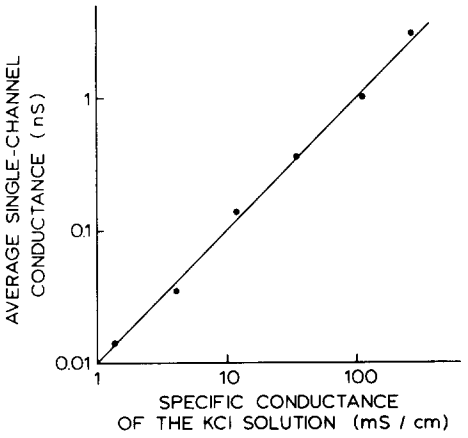

Fig. 4. Dependence of single-channel conductance on the specific conductance of solutions containing varying concentrations of KCl. The membrane was made with 2.5% glyceryl monooleate in *n*-decane over a hole of 0.1 mm^2. The 40 kDa protein was added to the aqueous phase at a final concentration of 0.3 ng/ml and the applied voltage was 50 mV. For each point, the average single-channel conductance was calculated for at least 200 steps, except at 10^{-2} M KCl, for which 52 steps were examined.

values of the single-channel conductances which we obtained for these proteins (2.0 and 1.3 nS, respectively) match those reported by Benz and co-workers [8].

A linear relationship was observed between average single-channel increments and the specific conductances of KCl solutions (Fig. 4). Such a result is expected for a large water-filled channel. Further evidence that the 40 kDa protein functions as a water-filled pore was obtained by measuring the single-channel conductance in the presence of ions of different size and charge (Table II). For a 13-fold variation in the average single-channel conductance (Λ) and a 9-fold variation in the specific conductance of the salt solutions (σ), the ratio of Λ to σ remained remarkably constant for monovalent salts. The single exception was for Na-Hepes. This ratio was only slightly smaller for salts of divalent ions and varied about 2-fold for all salts tested. These data indicate that the single-channel conductance of the 40 kDa protein matched reasonably well the conductance of the salt solutions bathing the membrane. These results suggest that the channels formed by the 40 kDa protein probably have very low selectivity for either cations or anions.

The pores formed by the 40 kDa protein were not voltage-gated since the average current per channel was directly proportional to the voltage applied (Fig. 5). The channels also appeared to be symmetrical, since switching the voltage from +20

TABLE II

AVERAGE SINGLE-CHANNEL INCREMENT OF THE 40 kDa PROTEIN IN DIFFERENT SALT SOLUTIONS

The pH of the salt solutions was between 6 and 7 if not indicated otherwise. The voltage applied was 20 mV and the membrane was formed with 2.5% glyceryl monooleate in *n*-decane over a hole of 0.1 mm^2.

Salt	Concentration (M)	Average single-channel conductance (Λ) (nS)	Specific conductance (σ) of the salt solution (mS/cm)	Λ/σ (10^{-8} cm)	Number of steps recorded
LiCl	1.0	0.89	71	1.3	274
NaCl	1.0	1.0	84	1.2	312
KCl	1.0	1.1	112	1.0	353
RbCl	1.0	1.4	115	1.2	215
NH_4Cl	1.0	1.2	106	1.1	210
$MgCl_2$	1.0	1.1	128	0.9	351
$CaCl_2$	1.0	1.2	156	0.8	250
$(NH_4)_2SO_4$	1.0	0.92	130	0.7	185
KCH_3COO	1.0	0.77	70	1.1	199
Tris-HCl	0.5	0.37	30	1.2	230
Na-Hepes (pH 9.0)	0.5	0.11	18	0.6	57

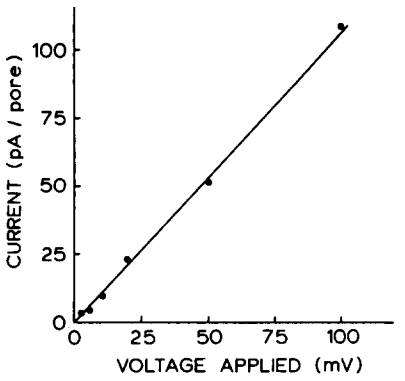

Fig. 5. Relationship between the average current through single channels and the voltage applied across the membrane. The aqueous phase contained 1.0 M KCl and 0.3 ng/ml of the 40 kDa protein. The membrane was made with 2.5% glyceryl monooleate over a hole of 0.1 mm^2. For each point, at least 200 steps were examined.

to −20 mV produced an equivalent current of opposite sign across the membrane.

Zero-current membrane potential measurements

After forming a membrane with at least 100 porin channels, zero-current membrane potentials were measured by increasing the salt concentration on one side of the membrane. Table I compares the membrane potential measured in the presence of the 40 kDa protein with those of a cation-selective porin (OmpF) and an anion-selective porin (NmpC) of *E. coli*; a 10-fold concentration gradient of KCl was employed. The values obtained for both OmpF and NmpC of *E. coli* are again similar to those reported by Benz and coworkers [8]. The selectivity ratio of the permeability of the cation to the permeability of the anion, P_c/P_a, was calculated with the Goldman-Hodgkin-Katz equation [18]. The value of 1.6 indicated that the 40 kDa protein of *H. influenzae* is poorly selective for K^+ over Cl^-.

Discussion

The results of the present study confirm our previous conclusion [14] and provide additional evidence that the 40 kDa protein of *H. influenzae* type b is a porin. The properties of its pores are similar to those of large, water-filled channels and are characteristic of most bacterial porins [1].

Pores formed by the 40 kDa protein were about 1.6-times more permeable to K^+ ions than to Cl^- ions. This very slight cation selectivity is similar to that of the porin of *Anabaena variabilis* [19] but smaller than those of all *E. coli* porins except PhoE and NmpC, which are anion-selective [8]. Our results may explain the higher permeability of the outer membrane of *H. influenzae* toward β-lactam antibiotics when compared with that of *E. coli* [13]. Many β-lactam antibiotics are anionic. The two porins usually present in the outer membrane of *E. coli* K-12, OmpF and OmpC, are cation-selective [8]. Nikaido and co-workers have shown, both in intact cells [20] and in reconstituted liposomes [9,21], that for *E. coli* porins, the charge carried by these compounds has a drastic effect on the rates at which they diffuse through the pores. Among the cephalosporins, anionic compounds diffused more slowly than zwitterionic compounds of comparable hydrophobicity. Anionic β-lactams also diffused more slowly through OmpC channels than through OmpF channels [20], the former being more cation-selective. In contrast, the charge of the antibiotic did not appear to influence significantly its rate of diffusion across the outer membrane of *H. influenzae* [13].

The magnitude of the single-channel increments produced by the 40 kDa protein of *H. influenzae* was much smaller than might have been expected from our determinations of the molecular weight exclusion limit [14]. Assuming the channel is a cylinder of constant cross-section, its radius can be calculated by the equation:

$$\Lambda = \frac{\sigma \pi r^2}{l} \quad (1)$$

A pore length (l) of 6 nm is the value assumed by the other workers [8] and is in agreement with the length of *E. coli* OmpF trimers as estimated from X-ray diffraction studies [22]. Based on the average single-channel conductance (Λ) of the 40 kDa protein in 1.0 M KCl (specific conductance, σ = 112 mS/cm), we calculated a pore diameter ($= 2r$) of 0.9 nm. This diameter is smaller than those estimated for any of the porins of *E. coli* [8]. On the other hand, the exclusion limit of about 1.4

kDa of the porin of *H. influenzae* [14] is clearly larger than the value of 600 Da obtained for the OmpF porin of *E. coli* [3], which has a single-channel conductance of 2.0 nS (Table I) [8].

This finding is rather surprising, since the 40 kDa protein was selected for detailed studies because, among the major outer membrane proteins of *H. influenzae*, it was the only one which rendered lipopolysaccharide-phospholipid vesicles permeable to oligosaccharides [14]. Eqn. 1 is based upon the assumption that the pores are filled with a solution of the same conductance as the external aqueous phase [1]. In agreement with this assumption, the single-channel conductance was a linear function of the specific conductance of the aqueous phase for the 40 kDa protein of *H. influenzae*. This result has been found for most bacterial porins [1]. Both Eqn. 1 and the Renkin equation [7] are based on the assumption that the pores are cylindrical. Structural studies of the OmpF porin trimers of *E. coli* have shown, however, that each trimer forms a single pore with three openings at the external surface of the outer membrane. These three channels apparently merge to form a single opening at the inner surface of the outer membrane [23]. More detailed knowledge of the shape of the pores will be required to understand the function of bacterial porins. In particular, it is not known if porins from different bacteria form pores of similar shape. It seems a likely possibility that porins of different bacteria could form channels with significantly different conformations.

To reconcile the large exclusion limit for the 40 kDa protein with its small single-channel conductance, we propose the following simple model. The pores formed by the porin of *H. influenzae* are straighter than those formed by *E. coli* porins (Fig. 6). Channels from both organisms are imagined as having a single opening on the periplasmic surface of the membrane and three openings on the external surface. For simplicity only two of these are shown in Fig. 6. Both channels have cylindrical segments but the diameter of these segments is slightly smaller in the pore formed by the 40 kDa protein of *H. influenzae*. Small ions cross both channels readily (Fig. 6A, D). If tested in the liposome swelling assay, the pores formed by the porin of *H. influenzae* appear larger, since small solutes such as glucose diffuse faster through

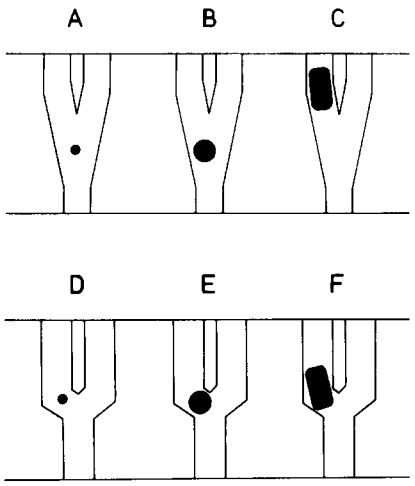

Fig. 6. Schematic representations of the structure of the channels formed by the 40 kDa protein of *H. influenzae* type b (A, B, C) and by the OmpF protein of *E. coli* (D, E, F) in a bacterial outer membrane of 6.0 nm thickness. Small ions are shown as small filled circles and traverse both channels at about the same rate (A, D). Small organic solutes such as glucose (larger filled circles) diffuse faster through pore B than through pore E. An oligosaccharide with an elongated structure such as stachyose (filled rectangular shapes) diffuses through pore C, but not through pore F. The diameter of pore A would then appear only slightly smaller than pore D when estimated from their channel conductances. Pores B and C, however, would appear larger than pores E and F when tested with the liposome swelling assay or the efflux assay.

this pore than through a more highly curved pore (Fig. 6B, E). Finally, the 40 kDa protein has a larger exclusion limit, since its structure accommodates the diffusion of rod-shaped molecules such as stachyose and longer unbranched oligosaccharides. These latter molecules are excluded from OmpF channels (Fig. 6C, F).

The shapes of the channels drawn in Fig. 6 differ from the recently published shape of the OmpF channel which was determined by electron microscopy and image reconstruction of negatively stained OmpF crystals [24]. The illustrations of Engel et al. [24] show a pore made of three stems which, taken two by two, form a V-shaped structure. The three openings on the same side of the membrane have a similar size. The single opening on the other surface of the membrane, however, is much larger. Its surface area appears to be comparable to the sum of the cross-sectional areas of the three individual stems of the channel

[24]. Such a shape is very difficult to reconcile with the functional properties of porins. If all three pores are open as the trimer inserts into the membrane, the single-channel conductance will depend on the smaller of either (a) the sum of the areas of the smallest cross-section within each of the three stems of the trimer, or (b) the area of the smallest cross-section within the common stem of the trimer. On the other hand, the exclusion limit of the pore will depend mostly upon the diameter of only one of the three stems of the pore. The diameter of OmpF channels should therefore appear much larger when estimated from their single-channel conductances. If the common stem of the OmpF channel has an area of about 1.1 nm^2, as calculated using Eqn. 1, the individual stems of the channel would have radii of about 0.34 nm. This value is smaller than the hydrated radii of arabinose (0.38 nm) or glucose (0.42 nm) [25] and could not account for the exclusion limit of this pore. We therefore conclude that all four stems of the channel should have similar, but not necessarily identical diameters.

Our model represents a simplification of pore structure and other possible explanations may account for our results. For example, we cannot exclude the possibility that purification procedures could cause a change in the structure of the pores which would be effective in lipid bilayers but not in liposomes because of the different reconstitution methods and use of different lipids. Our model emphasizes, however, the probable importance of pore structure in porin function and outer membrane permeability. Interestingly, mutants of OmpF and OmpC porins of *E. coli* have been isolated recently [26] which allow the diffusion of maltodextrins with an average molecular mass greater than 1 kDa. A change in the shape of the pores formed by these mutant porins could account for their increased molecular weight exclusion limits. A comparison of the pore diameter of these channels with their wild-type counterparts as estimated with the liposome swelling assay and by single-channel conductance experiments would provide a critical test of our model. Finally, since the pore size which is estimated by different methods can differ significantly, our results point out the need to study porins by a variety of experimental approaches.

Acknowledgements

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