Biochimica et Biophysica Acta, 1021 (1990) 175–181
Elsevier

BBAMEM 74708

Polarity-dependent voltage-gated porin channels from *Escherichia coli* in lipid bilayer membranes

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(Received 24 May 1989)

Key words: Porin; Montal-Mueller cell; Membrane reconstitution; Voltage gating

A porin preparation from *Escherichia coli* 0111 : B4 consisting of Omp F and Omp C (with Omp F in excess) was purified by salt extraction procedures and investigated in bilayer lipid membranes formed according to the Montal-Mueller technique. The porin preparation was added to the KCl electrolyte compartment of the Montal-Mueller cell which was connected to the voltage source. As the porin incorporated into the membrane, asymmetric, voltage-gated ion channels were formed. Transmembrane voltages > +50 mV (measured with respect to the side of porin addition) caused channel closing, while negative voltages, on the other hand, had no effect on channel behaviour but did increase the rate of porin incorporation at higher voltages. With porin added to both compartments voltage gating no longer occurred. Single-channel conductances corresponded to effective pore diameters of 1.5 nm for opening events and 1.18 nm for channel closing events. The number of charges involved in gating was approximately 2.

Introduction

The cell envelope of Gram-negative bacteria is composed of three layers: the cytoplasmic membrane, the periplasm containing proteins and peptidoglycan, and the outer membrane. The latter forms a barrier protecting cells from the detergent action of bile salts and degradation by hydrolytic enzymes [1]. In addition to this protective property, the outer membrane also acts as a molecular sieve permitting the passage of hydrophilic molecules with a size below a specific exclusion limit. One consequence of this permeability barrier function is that the entry into cells of many antibacterial agents, including some β-lactam antibiotics, is restricted or inhibited completely [2]. Small hydrophilic molecules cross the outer membrane by diffusion through water-filled trans-membrane channels formed by a class of proteins called porins. Porin channels, the dimensions of which determine the exclusion limit, have been identified and characterised in a large number of Gram-negative bacteria [3] and also the mitochondria and chloroplasts of eukaryotic cells [4].

Most bacterial porins studied to date show similar properties in that they form weakly anion selective channels which generally remain open irrespective of the transmembrane potential, i.e., they are voltage independent [5–7]. Some workers, however, have shown that porins from *Escherichia coli* form voltage-gated channels when incorporated into artificial bilayer membranes [8–10]. A large number of mitochondrial outer membrane porins have been shown to be gated by transmembrane voltages exceeding 35–40 mV [11], in contrast with applied voltages of > 140 mV [8,9] or > 100 mV [10] which were required to initiate gating of Gram-negative bacterial porins. In this paper we show that the voltage-dependent gating of bacterial porins can occur for voltages as low as 50 mV, and furthermore that such gating is dependent on the polarity of the applied potential.

Materials and Methods

Biomass preparation

Escherichia coli strain 0111 : B4 was incubated at 37 °C in tryptone soya broth (TSB; Oxoid CM129)

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0005-2736/90/$03.50

overnight with shaking. Cells were harvested in late exponential phase by centrifugation and resuspended in 10 mM phosphate buffer (pH 7.0).

Porin extraction and purification

Cells were disrupted by passage through a Manton-Gaulin homogeniser at 5000 psi, 5 ° C, and undamaged cells removed by centrifugation at 5000 × *g*. The supernatant was centrifuged at 200000 × *g* for 30 min and the cell envelope pellet resuspended in buffer and centrifuged as above. Pelleted material was resuspended in a minimum amount of buffer, the protein content estimated after Bradford [12], and the protein concentration adjusted to 10 mg/ml.

A sufficient volume of sodium lauryl sarkosinate (sarkosyl, 20% w/v) in 100 mM Tris-HCl (pH 8.0) was added to the cell envelopes to achieve a sarkosyl concentration of 2% w/v. The suspension was incubated at room temperature for 30 min with occasional mixing, and sarkosyl insoluble outer membranes collected by centrifugation at 200000 × *g* for 30 min. Outer membranes were washed as above and resuspended in buffer to a protein concentration of 4.5 mg/ml. Sodium dodecyl sulphate (SDS) was added to a final concentration of 2% w/v and the suspension incubated at 60 ° C for 30 min, after which the insoluble peptidoglycan-porin complex was harvested by centrifugation as before, washed in distilled water and recentrifuged. The pellet was resuspended in a small volume of 50 mM Tris-HCl (pH 8.0) containing 0.4 M NaCl, 1% w/v SDS, 5 mM EDTA, 3 mM NaN_3 and 0.05% mercaptoethanol and incubated at 37 ° C for 2 h with occasional mixing. Insoluble peptidoglycan was removed by centrifugation and the porin-containing supernatant mixed with an equal volume of acetone and kept at 4 ° C for 2 h. The precipitate was collected by centrifugation, washed with acetone and distilled water, then dissolved in 50 mM Tris-HCl (pH 8.0) containing the detergent lauryldimethylamine oxide (LDAO) 0.1% w/v to a final concentration of 2 mg/ml. This solution was further diluted with distilled water to a concentration of 100 μg/ml for use with bilayer membranes.

SDS-polyacrylamide gel electrophoresis

Quantitative evaluation of porins was performed using SDS-PAGE and staining the gels either with silver (for LPS and proteins) or Page blue 83 (for proteins only). The running buffer contained glycine (28.8 g/l), Tris (6.0 g/l) and SDS (1.0 g/l) in distilled water. Porins were examined in duplicate after incubating in gel sample buffer (125 mM Tris-HCl, 3.6% w/v SDS, 10% w/v mercaptoethanol, 20% w/v glycerol, 0.0001% w/v Bromophenol blue (pH 6.8); 100 μl sample plus 50 μl sample buffer) for 5 min, one sample at 100 ° C, the other at 20 ° C. Porin concentration was calculated using scanning laser densitometry of separated porin bands on a Page blue 83 stained SDS-PAGE gel by reference to bands representing known concentrations of bovine serum albumin.

Lipid bilayer membrane formation

Bilayers were formed across a hole in a thin PTFE sheet according to the techniques of Montal and Mueller [13]; a diagram of the experimental apparatus is shown in Fig. 1. The lipid used was diphytanoylphosphatidylcholine (Avanti Polar Lipids Inc., Pelham AL) dissolved in a 20 : 1 hexane/ethanol mixture. The bilayer bathing medium was 250 mM KCl containing 2.5 mM Hepes (pH 7.0). Porin was added to compartment A to which the transmembrane voltage was applied (Fig. 1). The current was measured via Ag/AgCl electrodes using a virtual earth operational amplifier incorporating a 10^8 Ω feedback resistor.

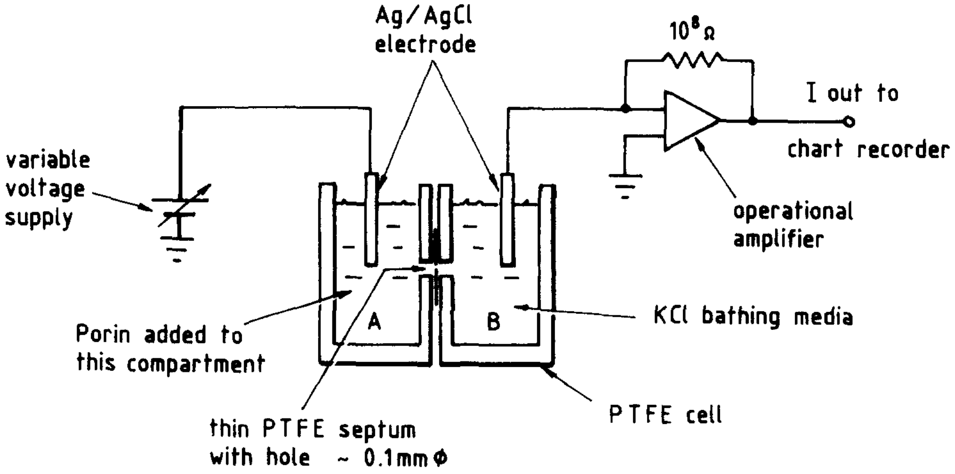

Fig. 1. Diagram of the experimental apparatus used to measure single-channel conductance of the porin preparation. Porin was added to compartment A to which the transmembrane voltage was also applied.

Results

Porin isolation

Examples of the stained SDS-PAGE gels are shown in Fig. 2. The sample incubated at 20 °C contained very little monomeric porin but large quantities of porin associated with lipopolysaccharide, track 1 [14]. The porin preparation incubated at 100 °C, however, (track 2) contained only denatured porins Omp F and Omp C, with Omp F in excess. The presence of the lipopolysaccharide was confirmed using silver staining (tracks 3 and 4) which showed ladder patterns typical of smooth type LPS. In this case porin monomers could not be visualised because Omp F does not stain with silver under these conditions. In this work no attempt was made to remove the LPS from the porin preparation.

Macroscopic conductance measurements

Having formed a stable bilayer membrane, porin was added to the bathing medium of compartment A of the cell to a final concentration of 1 μg/ml under an applied transmembrane voltage of either +50 or −50 mV. After about 5 min the current increased sharply by four orders of magnitude as shown in Fig. 3. Following this initial rapid rise which occurred over 2–3 minutes the current then increased slowly over the next 30–40 minutes, to around 10^{-7} A. This pattern of behaviour occurred for both positive and negative membrane potentials. Increasing the initial porin concentration increased the final steady state current until the membrane ruptured at typically 10^{-5} A. The effect of the detergent LDAO on the membrane was investigated by adding amounts ten times greater than used in any typical experiment to the bathing medium. No change in membrane resting current was noted after 30 min.

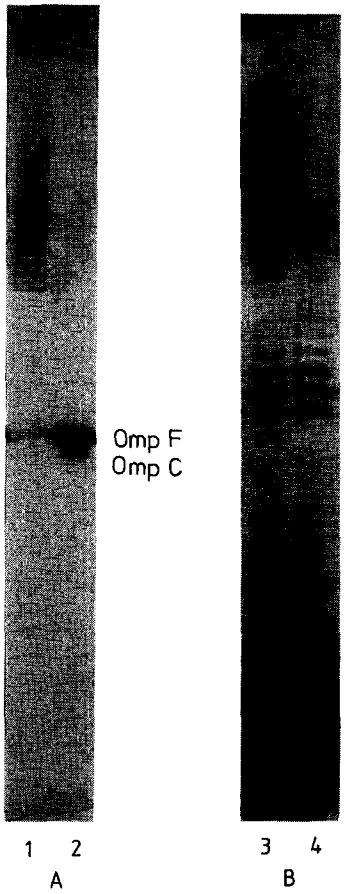

Fig. 2. SDS-polyacrylamide gels of porin preparations after electrophoresis and stained with Coomassie blue (A) and silver (B). Tracks 1 and 3 are porin samples heated at 20 °C, and tracks 2 and 4 porins heated at 100 °C.

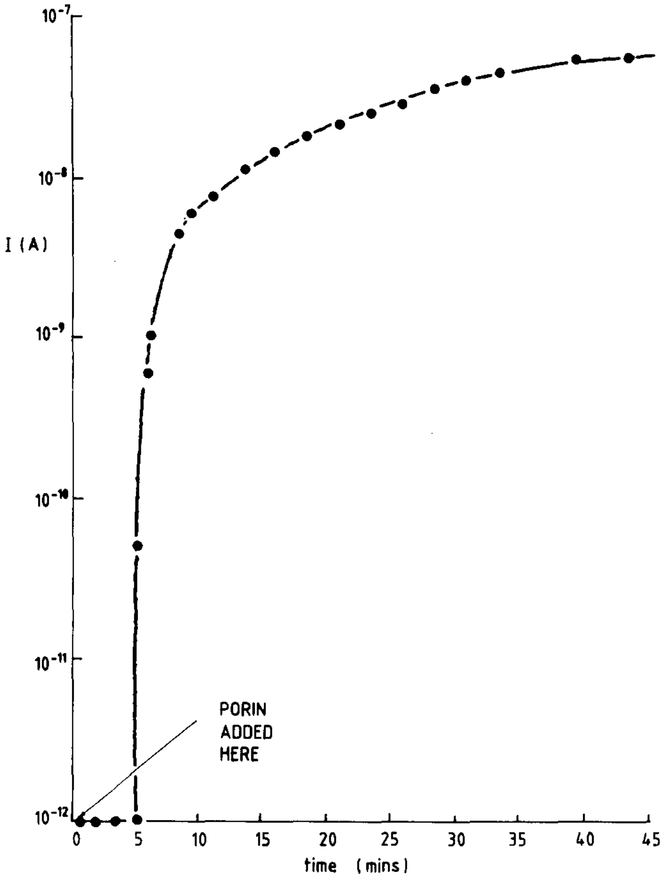

Fig. 3. Membrane current (I) at an applied transmembrane potential of +50 mV as a function of time. Porin was added to the aqueous phase of compartment A at time zero to a final concentration of 1 μg/ml. The membrane lipid was diphytanoylphosphatidylcholine and the bathing medium was 0.25 M KCl, 2.5 mM Hepes (pH 7.0), $T = 19$ °C.

Single-channel analysis

The presence of porin in the membrane caused the current to change in discrete steps as illustrated in Fig. 4. During channel insertion (rapid current rise in Fig. 3), and for applied transmembrane potentials of less than 50 mV most steps were directed in the increasing current direction. The opening and closing steps were of the form shown in Fig. 5, which illustrates discrete opening and closing events under an applied potential

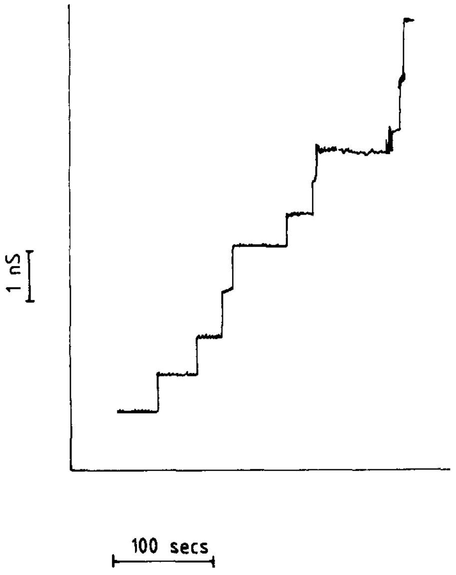

Fig. 4. Stepwise increase in membrane current after the addition of porin to the aqueous phase to a final concentration of 0.1 μg/ml. The membrane was formed from diphytanoylphosphatidylcholine and the applied voltage was +50 mV. (The resting current of the membrane without porin was 0.1 pA).

of +50 mV. This data, which was obtained from a membrane with 10 porin channels, also shows rapid (less than 1 ms) opening and closing events. These signals (identifiable as spikes on the recording shown in Fig. 5) were analysed with a storage oscilliscope and seen as square waveforms with time constants of typically 0.1 ms.

As is found for all transmembrane channels [5], the single conductance increments are not all of the same size but distributed over a range of values. A histogram of the probability of a channel having a particular conductance, Λ, as a function of channel conductance (obtained from approx. 200 single-channel events) under an applied potential of +50 mV is shown in Fig. 6. From this histogram it can be concluded that approx. 75% of the opening events occurred with a conductance of around 0.8 to 0.9 nS.

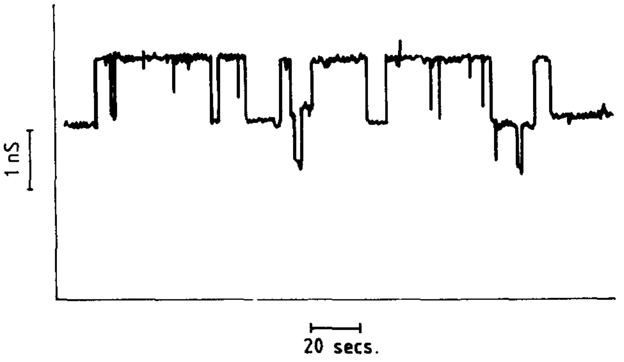

Fig. 5. Current trace of two channels at an applied potential of +50 mV showing discrete opening and closing events. The concentration of porin used was 0.1 μg/ml, and there were 10 channels present in the membrane at the time of this recording.

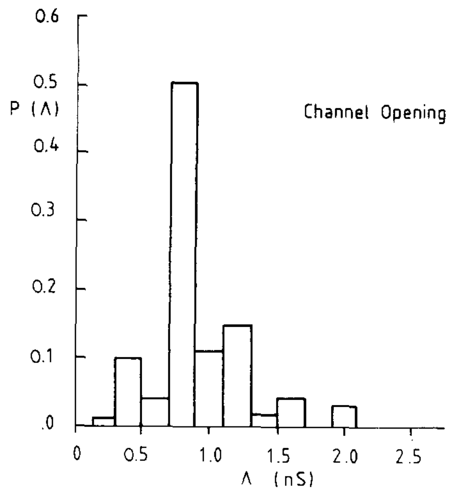

Fig. 6. Histogram of the conductance events due to channel opening for a porin doped membrane during porin insertion at an applied potential of +50 mV. It is apparent that 75% of channel opening events will have a conductivity of between 0.8 and 0.9 nS.

Voltage dependence

To investigate the dynamics of the pores, experiments were carried out on membranes that had stabilised with respect to porin insertion and had reached steady current levels. In the first experiment, the transmembrane voltages were set to zero, then increased to +10 mV for 5 s and the current recorded. The voltage

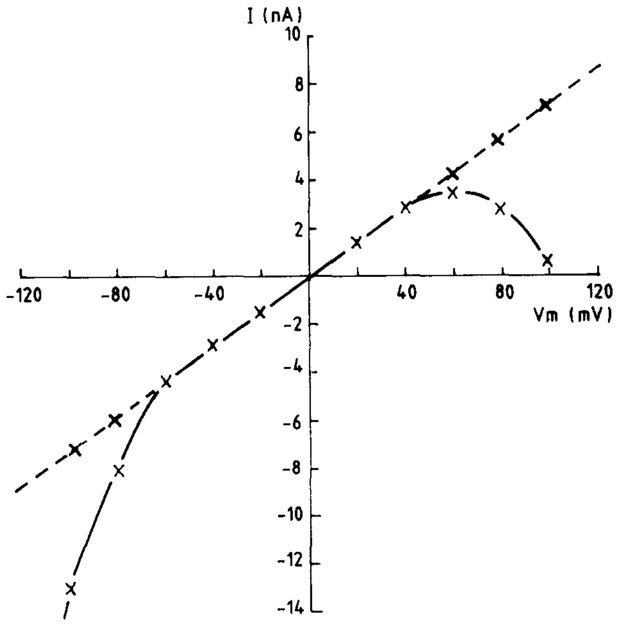

Fig. 7. Current (I) vs. voltage (V) plot for a porin doped membrane. Solid line represents 'steady-state' current. Dashed line represents the instantaneous current response which is ohmic and non-rectifying. The aqueous phase contained 0.1 μg/ml porin, 0.25 M KCl, 0.5 mM Hepes (pH 7.0), T = 19 °C.

was reset to zero, and after a short rest period of 5 s, set to −10 mV and the current recorded again. This pattern was repeated in voltage increments of ±10 mV. The resulting instantaneous current-voltage characteristic is linear in the range ±120 mV, as shown by the dotted line in Fig. 7.

The second experiment, also carried out on a stabilised membrane, was performed by increasing the voltage in steps of +10 mV from 0 to +120 mV and recording the current after approx. 100 s. This was repeated for negative potentials, i.e., 0 to −120 mV. Within the range ±50 mV the current flowing after 100 s was seen to be identical to the current flowing after 5 s. Significant differences occurred, however, when trans-membrane voltages in excess of 50 mV are applied where a marked polarity effect was observed; the current for positive voltages decreasing and for negative voltages increasing as the magnitude of the potential was increased.

Fig. 8 shows a typical current transient in the higher voltage regime when voltage steps of +80 mV and −80 mV were applied to the membrane. For positive voltages the current decreased with time to a steady-state value which was determined by the applied voltage. If, at any time during this decay, the membrane potential was briefly interrupted, the current dropped to zero and then returned to its *initial* value, as shown in Fig. 8. If, after a steady current had been attained, a reverse negative voltage was applied, then an instantaneous

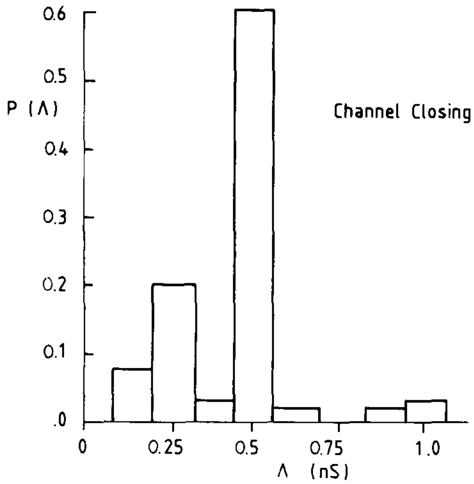

Fig. 9. Histogram of the conductance fluctuations observed for a porin doped membrane during closing at an applied potential of +80 mV. The channel closing events are seen to occur with a probability of 60% for a conductance level of approximately 0.5 nS, and a probability of 20% for a conductance level of 0.25 nS.

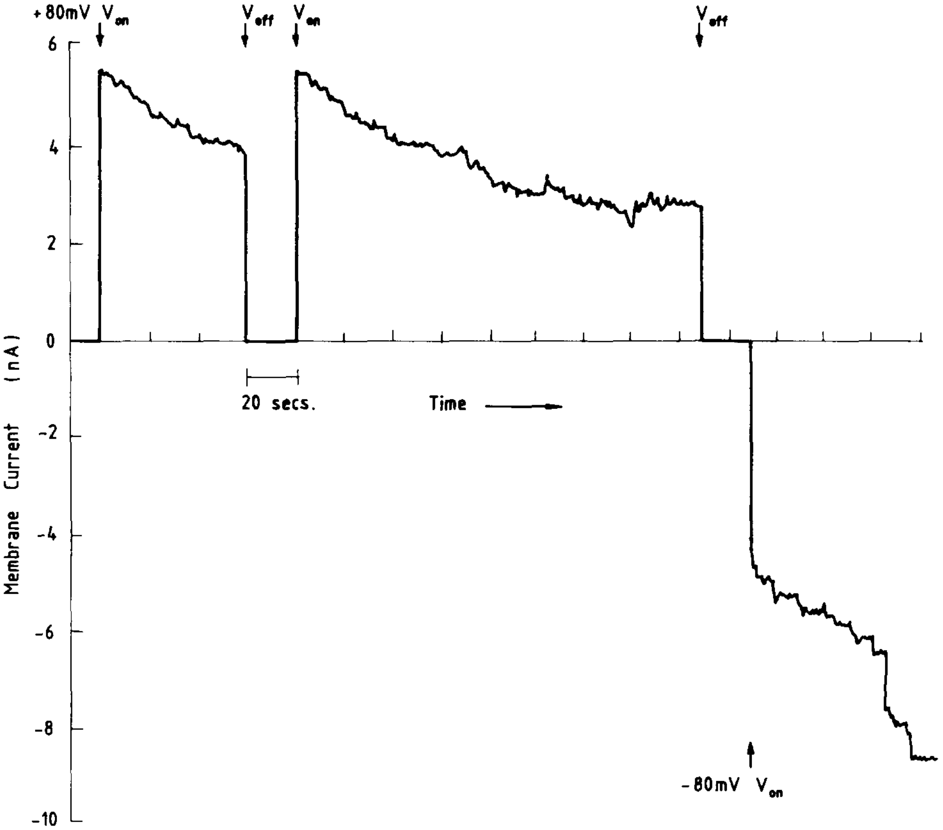

Fig. 8. Current trace of porin doped membrane after application of +80 mV to the membrane. The voltage was briefly et to zero and then returned to +80 mV after 60 s. After steady-state current levels had been reached the voltage polarity was reversed to −80 mV. Note relaxation of membrane current due to channel closing under positive potential. No channel closing is observed under negative potential, but field assisted porin insertion occurs. In all cases current change due to single-channel events can be resolved as small fluctuations in the current.

reverse current was obtained, equal in magnitude to the original instantaneous current. Thereafter, instead of decaying the current constantly increased.

The pattern of behaviour for positive voltages is characteristic of voltage dependent channel closing, and is similar to that reported by Schindler and Rosenbusch [8,9]. The reproducibility and fast time response of the current to voltage removal and reapplication excludes the possibility of channels being ejected from the bilayer. For negative voltages it is believed that the continuous increase in current is due to field-assisted porin insertion, and in many cases it was found that the rate of channel insertion was such that it caused eventual rupture of the membrane.

The asymmetry in the 'steady-state' current-voltage relationship shown in Fig. 7 (solid line) occurs irrespective of the sign of the applied potential during porin addition to the one compartment (comparment A). However, if porin is added to both compartments (A and B) then the steady-state current response shows no deviation from linearity for either positive or negative voltages.

Analysis of the single-channel behaviour of the closing events produces a histogram of the type shown in Fig. 9. Under an applied potential of +80 mV there is a 60% probability that a channel will close with a conductance of 0.5 nS and a 20% probability that a channel will close with a conductance of approx. 0.25 nS.

Discussion

The results presented show that the Omp F and Omp C porin from *E. coli* 0111 : B4 is able to form pores in artificial membranes in a manner similar to that reported for other Gram-negative bacterial outer membrane proteins. The conductance has been shown to vary in a discrete manner with a most probable single increment value of between 0.8 and 0.9 nS for a single channel opening event. Assuming that the pores are no more than channels filled with KCl of the same conductivity, σ, as the bathing medium (27.5 mS/cm for 250 mM KCl) then an estimate of the channel diameter ($d = 2r$) can be made from the equation:

$$\Lambda / \sigma = \pi r^2 / l \tag{1}$$

where Λ is the single-channel conductance and l is the channel length, assumed to be the bilayer thickness of about 6.0 nm. This gives a pore diameter of 1.5–1.6 nm for the peak of Fig. 6 which is in agreement with 1.6 nm reported by Xu et al. [10] and Lakey et al. [15] but larger than the diameter of 1.2 to 1.4 nm reported by Benz et al. [6,16] and 1 nm by Schindler and Rosenbusch [8,9].

The histogram of channel closing events (Fig. 9) is characterised by a large peak centred at 0.5 nS with a smaller peak at 0.25 nS. From Eqn. 1 the 0.5 nS peak predicts a main channel diameter of 1.18 nm for closing which is consistent with a value of 1.13 nm determined from the exclusion limit of various sugars (i.e., raffinose with a diameter of 1.128 nm) [17] and 1.16 nm from liposome swelling assays [18]. The magnitude of the probable conductance of the channel at closing is about two thirds of that at opening. This phenomenon is similar to that observed by Xu et al. [10] where an increase in the number of channel closing events at one third and two thirds the size of the major opening peak were observed as the applied transmembrane potential was increased.

From the data presented it is difficult to determine whether the main conductance of a channel arises from the trimeric form of the porin with a total channel conductance of 0.8 nS, or from the cooperative opening of three monomer porin channels each with a conductance equal to one third of the total. It is clear from comparison of Fig. 5 (data relating to channel opening at +50 mV) and Fig. 8 (data relating to channel closing at +80 mV), however, that high transmembrane voltages reduced the channel conductance.

Electron microscopy of Omp F shows that the porin appears as a triplet of holes set in the outer leaflet of the membrane [19,20] and that these holes lead to a single constriction of approx. 1 nm diameter in the periplasmic half of the membrane. Channel conductance will be controlled mainly by this constriction and as pointed out by Benz [4], closing one of the openings leading to this pore should reduce the total current by only 5% rather than 33%. Closing two openings should reduce the current by about 15–25% and not 66%. However, this is inconsistent with the results presented here. If high membrane voltages were to dissociate the trimer into active monomers, each capable of gating independently of the other then the one-third and two-third ratios in conductivity between opening and closing would follow.

Dissociation effects have been observed both in acidic bathing media and as a function of increasing membrane voltage [10]. Occassionally, [21,22] these were partly dependent on the detergent used to solubilise the sample.

Voltage-dependent gating can be analysed with a view to determining the number of gating charges involved [23,24]. The ratio of open channels to closed (N_o/N_c) at a given membrane potential V_m is given by the following equation:

$$N_o/N_c = \exp qn(V_m - V_o)/kT \tag{2}$$

where n is the number of charges moving through the membrane potential gradient, V_o is the potential for 50% of the channels closed (k = Boltzmann's constant, T = temperature, q = elementary charge and $kT/q = 25$

mV at room temperature). Plotting the data shown in Fig. 8 as log (N_o/N_c) against transmembrane voltage, V_m, yields a linear plot with a slope of 12 mV (± 1.5 mV) per e-fold change of N_o/N_c suggesting that approximately two charges are involved in the gating process. This compares with between 1 and 3 for mitochondrial porin [4,11]. Similarly V_o, the potential required to close 50% of the pores can be estimated to be 65 mV which also compares with that found for mitochondrial porins [11] but is substantially lower than that reported for any other voltage-dependent bacterial porin gating.

This result is somewhat surprising in that it suggests that Donnan potentials might well be capable of controlling the permeability of the Omp F pore. From work on reconstituting membranes from vesicles Schindler and Rosenbusch [8,9] showed that porins were gated and that the threshold for closing could be as low as 50 mV. They concluded that gating was thus likely to operate in intact cells. However, it has been pointed out that the porin may have been denatured when in monolayer form at the air/water interface [25], or that it did not fully penetrate the bilayer [16]. These arguments do not apply to the work presented here. In agreement with Schindler and Rosenbusch [8,9] the voltages required to initiate channel closing are found to be as low as 50 mV, but in contrast it is not necessary to apply a large voltage to initiate the first current responses.

The Donnan potential of the outer membrane of Gram-negative bacteria has been shown to vary from 5 to 80 mV (inside negative) in phosphate buffers ranging from 5 to 200 mM [26]. Recently Sen et al. [25] have measured the permeability of Omp F in intact cells as a function of the outer membrane Donnan potential by determining the influx rate of cephalosporins. They showed that the diffusion rate was not affected by a potential in the range of 5 to 100 mV, inside negative, and concluded that porin channels remain open in the presence of large membrane potentials.

Our measurements demonstrate an asymmetric voltage gating effect which possibly arises from the asymmetric addition of porins; this effect has also been noted for mitochondrial porins [5]. Furthermore, the porin is not a symmetric molecule and in our preparations is assumed to be bound to LPS. If the LPS is located near the outer face of the porin trimer then this will determine its orientation when inserted into a bilayer; the hydrophilic polysaccharide component remaining in the aqueous bathing medium. It is likely that the porin will enter the bilayer in a fixed orientation with the three pores on the outside, as has been suggested from electron microscopy [19,20]. In our experiments therefore, compartment A would correspond to the outside of the cell; all voltages were measured with respect to this side. It has been shown in this work that only potentials $> +50$ mV close the reconstituted pores and as such the equivalent Donnan potential required for closing, in vivo would be > 50 mV, inside negative, suggesting that Donnan potentials may well be capable of gating the porins.

Acknowledgements

This work was supported by the Science and Engineering Research Council. We thank Dr Martin Taylor for critical reading of the manuscript and Dr. Lindsay Bashford, Mr. S. Elson, Prof. T.J. Lewis, Prof. C.A. Pasternak and Dr. B.N. Zaba for their contributions.

References

1 Nikaido, H. and Vaara, M. (1985) Microbiol. Rev. 49, 1–32.
2 Nayler, J.H.C. (1987) J. Antimicrob. Chemother. 19, 713–732.
3 Hancock, R.E.W. (1987) in Bacterial Outer Membranes as Model Systems (Inouye, M., ed.), pp. 187–225, John Wiley & Sons, New York.
4 Benz, R. (1985) CRC Crit. Rev. Biochem. 19, 145–190.
5 Benz, R. and Bauer, K. (1988) Eur. J. Biochem. 176, 1–19.
6 Benz, R., Janko, K., Boos, W. and Läuger, P. (1978) Biochim. Biophys. Acta 511, 305–319.
7 Benz, R., Janko, K. and Läuger, P. (1979) Biochim. Biophys. Acta 551, 238–247.
8 Schindler, H. and Rosenbusch, J.P. (1978) Proc. Natl. Acad. Sci. USA 75, 3751–3755.
9 Schindler, H. and Rosenbusch, J.P. (1981) Proc. Natl. Acad. Sci. USA 78, 2302–2306.
10 Xu, G., Shi, B., Estelle, J.M. and Tien, H.T. (1986) Biochim. Biophys. Acta 862, 57–64.
11 DePinto, V., Ludwig, O., Krause, J., Benz, R. and Palmieri, F. (1987) Biochim. Biophys. Acta 894, 109–119.
12 Bradford, M.M. (1976) Anal. Biochem. 72, 248–254.
13 Montal, M. and Mueller, P. (1972) Proc. Natl. Acad. Sci. USA 69, 3561–3566.
14 Garavito, R.M. and Rosenbusch, J.P. (1986) Methods Enzymol. 125, 309–328.
15 Lakey, J.H., Watts, J.P. and Lea, E.J.A. (1985) Biochim. Biophys. Acta 817, 208–216.
16 Benz, R., Hancock, R.E.W. and Nakae, T. (1982) in Transport in Biomembranes: Model Systems and Reconstitution (Antonlini, R. et al., eds.), Raven Press, New York.
17 Nakae, T. (1976) Biochem. Biophys. Res. Commun. 71, 877–884.
18 Nikaido, H. and Rosenberg, E.Y. (1983) J. Bacteriol. 153, 241–252.
19 Dorset, D.L., Engel, A., Massalski, A. and Rosenbusch, J.P. (1984) Biophys. J. 45, 128–129.
20 Engel, A., Massalaski, A., Schindler, M., Dorset, D.L. and Rosenbusch, J.P. (1985) Nature (London) 317, 643–645.
21 Markovic-Housley, Z. and Garavito, R.M. (1986) Biochim. Biophys. Acta 869, 158–170.
22 Schindler, M. and Rosenbusch, J.P. (1984) FEBS Lett. 172, 85–89.
23 Hodgkin, A.L. and Huxley, A.F. (1952) J. Physiol. (London) 116, 424–448.
24 Hille, B. (1984) Ionic Channels of Excitable Membranes, Sinauer Assoc. Inc., Sunderland, MA.
25 Sen, K., Hellman, J. and Nikaido, H. (1988) J. Biol. Chem. 263, 1182–1187.
26 Stock, J.B., Rauch, B. and Roseman, S. (1977) J. Biol. Chem. 252, 7850–7861.