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Successful recovery of the normal electrophysiological properties of PorB (Class 3) porin from *Neisseria meningitidis* after expression in *Escherichia coli* and renaturation

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Abstract

Neisseria meningitidis PorB class 3 porins obtained either from native membranes (wild-type) or recovered from inclusion bodies following expression in *Escherichia coli* (recombinant), have been reconstituted into solvent-free planar phospholipid membranes. The wild-type and recombinant porins exhibited the same single-trimer conductance (1–1.3 nS in 200 mM NaCl), tri-level closure pattern, characteristic of functional channel trimers, and pattern of insertion into planar membranes. Both proteins were open at low voltages and displayed two voltage-dependent closure processes, one at positive and the other at negative potentials. Both showed asymmetric voltage dependence such that one gating process occurred at lower voltages ($V_o = 15$ mV) than the other ($V_o = 25$ mV). The sign of the potential that resulted in closure at low voltages varied from membrane to membrane indicating that they may have the property of auto-directed insertion (in analogy to the mitochondrial channel, VDAC). In the case of the recombinant porin, the steepness of the voltage dependence of one gating process was slightly less ($n = 1.3$) than that observed for the other process or for the wild-type channel ($n = 1.5$–1.7). Both channels have a high (40%) probability of closure even at 0 mV. While both channels show a slight selectivity for Cl^- over Na^+, the selectivity of the recombinant porin is a bit higher (permeability ratio of 2.8 vs. 1.6) as measured using a 2-fold salt gradient. Thus, the method employed to refold the recombinant porin was successful in not only restoring wild-type structure [H.L. Qi, J.Y. Tai, M.S. Blake, Expression of large amounts of Neisserial porin proteins in *Escherichia coli* and refolding of the proteins into native trimers, Infect. Immun. 62 (1994) 2432–2439; C.A.S.A. Minetti, J.Y. Tai, M.S. Blake, J.K. Pullen, S.M. Liang, D.P. Remeta, Structural and functional characterization of a recombinant PorB class 2 protein from *Neisseria meningitidis*. Conformational stability and porin activity, J. Biol. Chem. 272 (1997) 10710–10720] but also the overall electrophysiological function. © 1998 Elsevier Science B.V.

Keywords: Porin; Electrophysiological property; Recombinant; Voltage-gating; Outer membrane; (*Neisseria*)

1. Introduction

The outer membrane of Gram-negative bacteria contain aqueous-channel forming proteins collectively referred to as porins [see Ref. [3] for review]. The channel characteristics of these porin proteins are

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generally elucidated electrophysiologically after reconstitution into planar phospholipid membranes. The porins studied to date have some basic characteristics in common. Each functional unit, the trimer, closes in 3 discreet steps corresponding to the closure of each of the 3 channels. This closure is generally voltage dependent although the amount of applied voltage required to get closure varies widely. Most of the major porin proteins prefer the passage of cations but are not absolutely selective for cations over anions [4,5]. In contrast, porins from *Neisseria gonorrhoeae* have been shown to be more selective for anions [5–8]. There are also a group of specialized porins such as PhoE which show distinct specificity [9,10].

Biochemical and structural studies of membrane proteins often require access to large quantities of pure, homogeneous, and well-defined proteins. Bacterial expression systems are useful at producing large quantities of soluble proteins but heterologous or overproduced membrane proteins almost invariably are toxic or end up in inclusion bodies. The restoration of native structure and function to proteins isolated from inclusion bodies often represents a challenge. Many attempts in obtaining native-like porins following isolation from inclusion bodies have met with limited success [11–14]. Even when channel function is restored, the electrophysiological properties are often very different from the wild-type protein. Reconstituted *Haemophilus influenza* type b porin had a somewhat lower conductance than the wild-type and the histogram was broader, indicating more heterogeneity [13]. Further studies of the recombinantly produced *Haemophilus* porin showed effective channel formation even with misfolded proteins. Likewise, the native Oms28 porin of *Borrelia burgdorferi* forms primarily 0.6 nS channels in 1 M KCl but when the recombinant protein was tested, its conductance was 1.1 nS and the channel-forming potency was reduced by 15–45 fold [14]. Hence, careful characterization of a variety of electrophysiological properties as well as structural properties are required to ensure that the recovered proteins are in a structure approaching the native state. Here, we report the successful recovery of function of a porin from *Neisseria meningitidis*, to a degree that it is virtually identical to that of the wild-type protein.

Meningococcal porins form channels in the outer membrane of *N. meningitidis* [6]. Like other porins, they exist as trimers, each monomer forming an ion-conducting pathway. As opposed to *N. gonorrhoeae* which has only one porin gene, meningococci can have two genes for porin expression. The *PorA* gene is responsible for the production of the formerly known Class 1 protein. The presence of the Class 1 porin varies, depending on the strain and/or isolate. Most of the porins found in meningococci originate from the *PorB* gene which has two major mutually exclusive alleles, one expressing the family of Class 2 porins and the other the Class 3 porins [15,16]. Initial attempts to express Neisserial porins in *E. coli* were limited, with several studies showing that even minute quantities of porin expression being lethal for the heterologous host [17–19]. However, Qi et al. found that by replacing the signal sequence of the Neisserial gene with that of Φ10 and placing this behind the T7 promoter, large amounts of these porin proteins could be produced in the form of inclusion bodies [1]. A similar strategy was used to efficiently express class 1 porin in *Bacillus subtilis* [20]. A method was developed to restore these recombinant Neisserial porins to their native trimeric form [1,2].

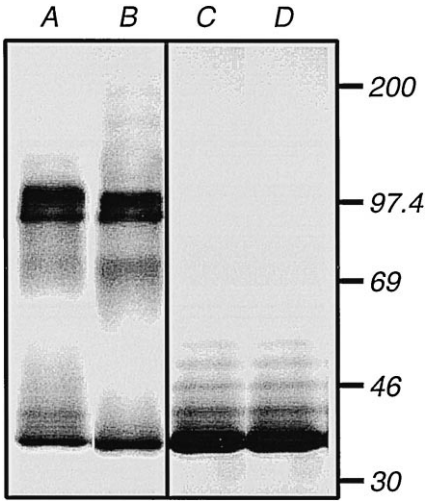

Fig. 1. An SDS-PAGE analysis of wild-type native and recombinant PorB/C3. Native wild-type PorB/C3 (lanes A and C) was extracted from meningococcal strain 44/76 Δ1Δ4 and purified as described in Section 2. The recombinant rPorB/C3 (lanes B and D) was overexpressed in *E. coli* BL21 [DE3] Δ*ompA*, isolated, refolded, and purified as described in Section 2. Samples in lanes A and C were treated with 0.25% SDS and thus retain some trimeric forms. Samples in lanes C and D were treated with 1% SDS resulting in only monomers.

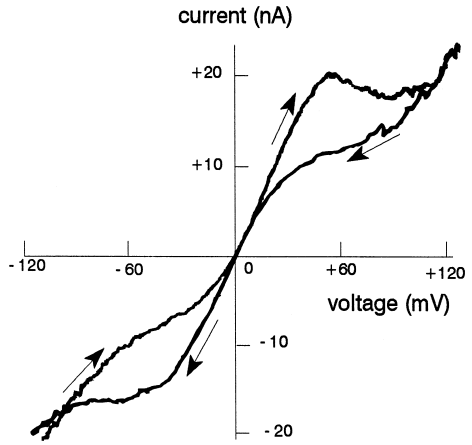

Fig. 2. The voltage dependence of current flow through a membrane containing many wild-type PorB/C3 channels. A triangular voltage wave ranging from −120 mV to +120 mV was applied at a frequency of 5 mHz. What is illustrated is the current recorded during one complete cycle as a function of the applied voltage. The trace begins at the lower left (−120 mV), proceeds to the upper right (+120 mV) and end at the lower left. The arrows indicate the temporal direction of the recording. The portions of this record that would be used for analysis of the voltage dependence or the generation of conductance/voltage plots are from −120 mV to +20 mV and from +120 mV to −20 mV. This is the direction in which the channels are opening and where the system is in quasi-equilibrium between the available states.

Qi et al. demonstrated that these refolded trimeric porin proteins were immunologically the same as the porins produced in *Neisseria* species [1]. Additional studies showed that these recombinant Neisserial porins are virtually identical, both biochemically and structurally, to the wild-type form [2]. To characterize more exactly these refolded Neisserial porins, class 3 porins was examined electrophysiologically to look for functional differences between the recombinant and wild-type forms. As only a brief report on the electrophysiological properties of meningococcal porins exists in the literature [6], a more careful characterization of both the wild-type and the recombinant form was undertaken.

2. Materials and methods

2.1. Bacterial strains, growth conditions, and reagents

The solid typing media and liquid growth media for all the meningococci have been previously described [21]. The meningococcal strain 44/76 (Δ1Δ4) were provided by Dr. Lee Wetzler, Maxwell Finland Laboratory, Boston City Hospital, Boston, MA and has been described previously [22]. The growth media and conditions for the preparation of *E. coli*, strain BL21[DE3]Δ*ompA*, harboring the expression plasmid pNV15 was similar to that of Qi et al. [1]. All reagents not specifically described were from Sigma (St. Louis, MO) and of the highest grade possible.

2.2. Isolation and purification of porins

PorB class 3 (PorB/C3) protein from meningococcal strain 44/76 (Δ1Δ4) was isolated employing the zwittergen–Ca^{+2} extraction procedure described by Wetzler et al. [23]. The procedure employed for the over expression, isolation, refolding, and purification of PorB class 3 protein (strain 44/76) in *E. coli* (rPorB/C3) was similar to that described elsewhere [1,2]. Protein concentration was estimated by measuring the absorbance at 280 nm, employing a HP

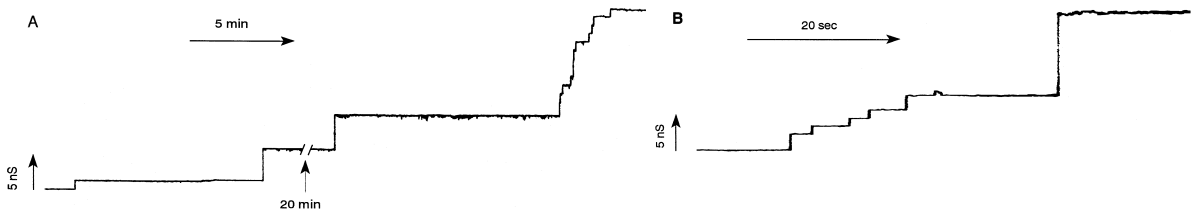

Fig. 3. Insertion of wild-type (panel A) and recombinant (panel B) PorB/C3 channels into planar phospholipid membranes. A sample (5 μl) of detergent solubilized porin was stirred into the aqueous phase (200 mM NaCl) bathing the phospholipid membrane. The voltage was clamped at 6 mV (A) or 8 mV (B) and after a variable delay, the conductance increased as shown. Both single and multi-trimer insertion events were observed.

Model 8453 UV/Vis rapid scan spectrophotometer equipped with a diode array detector (Hewlett-Packard, Palo Alto, CA), using a molar extinction coefficient of 41,960 which was calculated based on class 3 aromatic amino acid content according to Mach et al. [24]. The identity of the protein sequences in the wild-type (PorB/C3) and recombinant (rPorB/C3) protein was checked by sequencing the genes.

Fig. 1 shows the SDS-PAGE analysis of both the purified wild-type and the recombinant used in our studies. Samples were prepared in 10 mM phosphate buffer and 0.05% Z 3–14 were loaded into a 8–16% Tris glycine gel (Novex) following incubation with either 0.25% SDS (lanes A and B) or 1% SDS (lanes C and D). These porins are very sensitive to SDS and easily dissociate into monomers. Thus, in 0.25% SDS, both trimers and monomers are evident but in 1% SDS, only monomers are present.

2.3. Channel reconstitution

Solvent-free planar phospholipid membranes were produced by a modification [25] of the monolayer method of Montal and Mueller [26]. Diphytanoylphosphatidylcholine (Avanti Polar Lipids, Alabastor, AL)/cholesterol (Sigma) 5:1 was used to make the membranes and the medium was generally 200 mM NaCl. The membrane was voltage clamped and calomel electrodes were used to interface the electronics with the aqueous phase [25].

Channels were inserted by adding, a 5 μl aliquot of detergent-solubilized PorB/C3 to a 5 ml aqueous phase bathing the membrane while stirring. No channel insertion was observed unless the sample was supplemented with Triton X100 to a final concentration of 1% (v/v) prior to addition of the 5 μl aliquot to the chamber. The final concentration of Triton in the chamber was 0.001% (v/v). The Triton requirement was the same for both wild-type and recombinant protein. Channel insertion was delayed until a first event occurred and then proceeded in an erratic but progressive manner.

2.4. Analysis of the voltage dependence

The reconstituted channels showed very slow and voltage-dependent rates of opening and closure. Thus the strategy used to quantitate the voltage dependence was one originally developed for the mitochondrial channel, VDAC (also referred to as mitochondrial porin) [27]. Slow triangular voltage waves were used to vary the voltage gradually and measure the resulting current through the channels in the membrane.

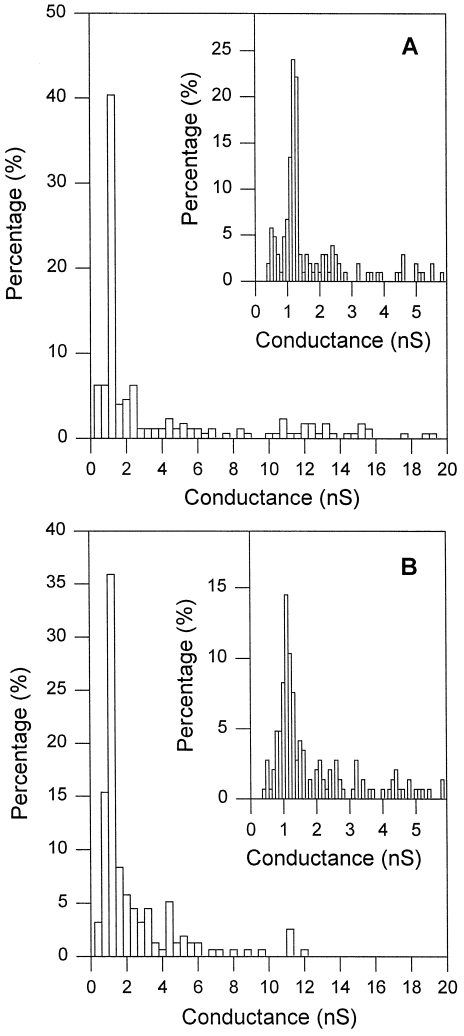

Fig. 4. Histograms of the size of discrete conductance increments associated with the insertion of PorB/C3 channels into planar phospholipid membranes. The conductance of insertion events such as those illustrated in Fig. 2 were measured and pooled from many experiments. The main figures show all observed events grouped into 0.4 nS bins while the insets show only the events up to 6 nS in magnitude grouped in 0.1 nS bins. The magnitude of the bars represents the percent of all insertions included in the figure that are in the particular conductance range. The wild-type channels are summarized in panel A and the recombinant channels in panel B.

When the voltage was varied between ±120 mV at a frequency of 5 mHz, hysteresis was evident (Fig. 2). Using a lower frequency (2 mHz) revealed that the kinetically-delayed curve was that recorded in the direction of increasing electric field where the channels were being closed. The other curve did not change indicating that, in the opening direction, the channels were achieving an equilibrium between states or at least a local equilibrium. Thus, further analysis was limited to the recording taken in the direction of channel reopening.

In order to obtain values for the voltage-dependence parameters that define the characteristics of the voltage dependence of each of the two gating processes, the data was fitted to a Boltzmann distribution. Each gating process is taken as an interconversion of channels between a closed and an open state. In this case, the closed state is a low-conducting state. At any voltage, an equilibrium is established between the two states that depends on the energy difference between the states. The portion of the energy difference that is voltage dependent is set equal to nFV_0, where n is a measure of the steepness of the voltage dependence and equal to the number of charges that would have to cross the entire potential difference in order to account for the observed voltage dependence, and V_0 is the voltage at which the channel has equal probability of being in either conformation (F is Faraday's constant).

The current recorded when the channels were reopening (high field to low field) was digitized and converted to a conductance by dividing by the applied voltage. Due to the presence of significant conductance in the closed state, the following version of the Boltzmann distribution was used:

$$\ln\left(\frac{G_{max}-G}{G-G_{min}}\right)=\frac{nF}{RT}V-\frac{nF}{RT}V_0$$

where G_{max} is the maximum conductance, G_{min} is the minimum or voltage-independent conductance, V is the transmembrane voltage and RT is the product of the gas constant and the absolute temperature. The slope and intercept yielded the values of n and V_0.

The two gating processes are sufficiently close on the voltage scale for their effects to overlap. As the voltage is slowly decreased, channels reopen due to one gating process but then should begin to close because of the other gating process. However, as a consequence of the slow rates of closure, only the first gating process influences the probability of the channel being open. Therefore the current records only reflect the action of one gating process. However, in vivo, the channels can reach equilibrium over long periods of time. Thus, we calculated the open probability (P_{open}) using the experimentally-determined n and V_0.values for both gating process:

$$P_{open}=\frac{N_{open}}{N_{open}+N_{closed(+)}+N_{closed(-)}}$$

$$P_{open}=1\Big/\left\{1+\exp\left(\frac{n_{(+)}F(V-V_{0(+)})}{RT}\right)+\exp\left(\frac{n_{(-)}F(V_{0(-)}-V)}{RT}\right)\right\}$$

where N_{open}, N_{closed}, are the number of channels in the open and closed states and the subscripts (+) and

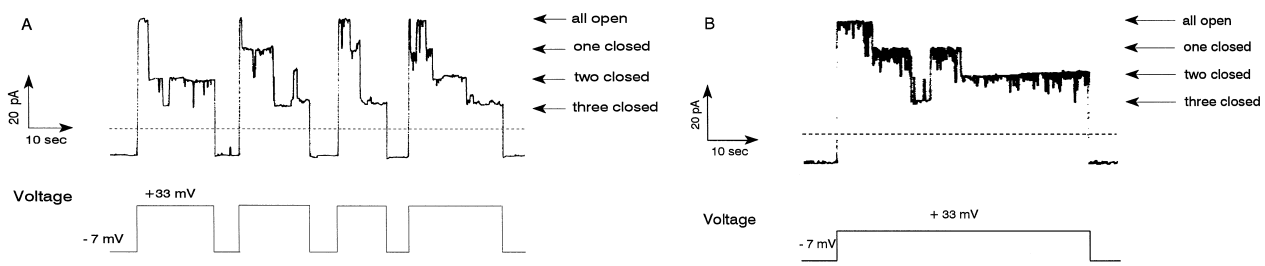

Fig. 5. Single 1.1 nS insertion event closed in three stages. In panel A, the closing properties of one wild-type PorB/C3 insertion event was examined by applying 4 successive pulses to higher voltages. The channel closed in 3 steps but the closing pattern varied from experiment to experiment. In panel B, a single recombinant PorB/C3 insertion event was tested as in panel A.

(−) refer to the positive and negative gating processes, respectively. This equation was used to generate the solid lines in Fig. 5.

3. Results

Detergent-solubilized PorB/C3 channels spontaneously inserted into planar phospholipid membranes resulting in discrete increases in the membrane's permeability to ions (Fig. 3). In both the wild-type and recombinant forms, the increases appeared to be multiples of a unitary conductance, one channel-forming trimeric structural unit. The size of this unit is essentially the same as that previously reported [6], 1–1.3 nS in 200 mM NaCl. The histograms (Fig. 4) for both the wild-type (Fig. 4A) and recombinant protein (Fig. 4B) showed the same level of polydispersion indicating either that similar levels of aggregation take place in solution following dilution of the detergent within the aqueous chamber or the same cooperative insertion process occurs. Finally, the potency of the two sources of protein was indistinguishable indicating the same level of channel-forming activity per unit amount of protein in the sample.

As is the case for all the porins, each channel closes in 3 distinct levels reflecting the trimeric pore structure (Fig. 5). The smaller insertional events (less than 1 nS) seen in Fig. 4 could be due to the insertion of trimers with 1 or 2 channels closed. The pattern of closure varied from experiment to experiment. Often two and sometimes all three closure events occurred almost simultaneously. Thus, some cooperativity in the voltage-dependent closing process is evident as previously seen in other porins [8,28]. Approximately, the same level of cooperativity was observed in both the wild-type and recombinant protein. The trace illustrated in Fig. 5B shows more fluctuations than that in Fig. 5A. This varied from channel to channel for both wild-type and recombinant proteins.

The voltage-dependence of PorB/C3 was quantitated by a method originally developed for VDAC [27]. Slow (5 mHz) triangular voltage waves were applied to a membrane containing hundreds of channels and the current was monitored. In a multi-channel membrane, the conductance drops at both positive and negative potentials (Fig. 6). But we know from observations with single channels that each channel closes at both positive and negative potentials. Thus, there are 2 gating processes resulting in 2 different closed states. Pronounced hysteresis was observed for

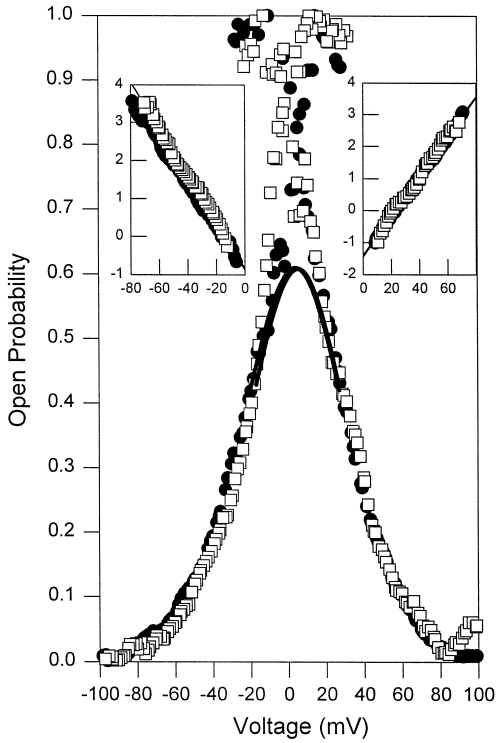

Fig. 6. The voltage-dependent properties of PorB/C3 porins. The figures illustrate 2 experiments, one performed on wild-type channels (filled circles) and the other on recombinant channels (open squares). The voltage dependence of the porins was measured by applying a 5 mHz triangular voltage wave whose voltage ranged from −120 mV to +120 mV. The current recorded when the channels were reopening (high field to low field) was digitized and converted to a conductance by dividing by the applied voltage. The voltage-independent conductance was subtracted and then the data was normalized by dividing by the maximal conductance in each case. These are the points plotted in the main figure. The magnitude of each point is a measure of the open probability, considering only one closing process assuming no occupancy in the other closed state (see Section 2). The insets show the fits to the Boltzmann distribution for the gating processes at positive and negative potentials. From the values of n and V_0 obtained (wild-type: $n(+)=1.6$, $n(-)=1.4$, $V_0(+)=23$ mV, $V_0(-)=15$ mV; recombinant: $n(+)=1.7$, $n(-)=1.5$, $V_0(+)=24$ mV, $V_0(-)=13$ mV), the probability of the channels being open considering both gating processes was calculated (see Section 2): This is shown by the solid curve in the middle of the figure, one curve for wild-type and one for the recombinant porin.

Table 1
Comparison of voltage dependence parameters

	Closure at lower potentials			Closure at higher potentials		
	n	V_0 (mV)	ΔG (kJ/mol)	n	V_0 (mV)	ΔG (kJ/mol)
Wild-type	1.58 ± 0.10 (7)	14.9 ± 1.0	2.27	1.54 ± 0.11 (7)	24.5 ± 2.7	3.64
Recombinant	1.69 ± 0.23 (4)	14.8 ± 0.3	2.41	1.32 ± 0.12 (6)	24.5 ± 1.5	3.12

The steepness of the voltage dependence (n) and the voltage at which half the channels are closed (V_0) were calculated from data such as that illustrated in Fig. 5. The values are means $\pm$ SD (no. of different membranes). The voltage-independent free-energy difference between the open and closed state for each gating process, ΔG, was obtained by multiplying the average n with the corresponding V_0 and with the Faraday constant.

both gating processes (Fig. 2) reflecting the slow rates of channel closure. Channel reopening is very fast, much faster than the response time of the chart recorder (0.3 s full scale deflection). The portion of the record collected while the channels were reopening (the electric field was being reduced) was not influenced by reducing the frequency to 2 mHz and so this portion was used to describe the voltage dependence of the channels and to calculate the voltage-dependence parameters, n and V_0 (see Section 2).

An example of the voltage-dependence of the conductance is shown in Fig. 6. The voltage dependence of the conductance during the reopening phase of the recording was plotted on Fig. 6. Thus, the right half of the figure was recorded by beginning at high positive potentials and slowly reducing the voltage (2.26 mV/s) to zero and a bit beyond (similarly for negative potentials). In this way, only the properties of one gating process was examined at one time. Since both gating processes should function at the same time, the probability of being open is less (calculated lines).

The data for each gating process were analyzed by fitting to a Boltzmann distribution between 2 states (see Section 2). Excellent fits were obtained (insets to Fig. 6). The voltage at which half the channels are closed, V_0, and the steepness of the voltage dependence, n, are the parameters obtained from these fits. These characterize the voltage dependence and are summarized, for various experiments, in Table 1. The channels show a clear asymmetry in that channel closure at one sign of the potential occurs at lower voltages than at the opposite sign ($P < 0.001$). The free energy difference between one closed state and the open state is 2/3 of that for the other closed state (Table 1). The sign of the voltage that resulted in closure at lower potentials varied randomly from membrane to membrane indicating that the orientation of the channels also varied in this way. In summarizing the data, the properties of the gating process that closed at lower voltages were averaged (similarly for those closing at higher potential). The results are almost the same for the wild-type and recombinant channels (Table 1). The only small, but statistically significant, difference was a lower n-value (1.32) for the recombinant channels for the high-voltage gating process. It is less than the corresponding wild-type value ($P < 0.005$) and the other n-value for the recombinant protein ($P < 0.01$), but not by much.

The free energy difference between the open and closed states (ΔG) can be calculated from the voltage dependence parameters. It is equal to nFV_0 which is the voltage-dependent energy needed to compensate for the conformational energy difference between the states. The energy differences were the same for wild-type and recombinant channels.

Ion selectivity was assessed by recording the zero-current potential in the presence of a 2 fold salt

Table 2
Comparison of ion selectivity

Protein source	Reversal potential (mV)	$P(Cl^-)/P(Na^+)$
Wild-type	3.5 ± 0.1 (3)	1.6
Recombinant	7.6 ± 0.2 (3)	2.8

The salt solutions were 0.2 M NaCl vs. 0.1 M NaCl (activity ratio is 1.89). The indicated reversal potential values are means $\pm$ SD (no. of estimates) and refer to the high salt side. The estimation of the ion permeability ratio was calculated using the Nernst–Planck equation and thus assumes electroneutrality.

gradient (200 mM NaCl vs. 100 mM NaCl). The Nernst Planck equation was used to estimate the ratio of permeability of Cl^- to Na^+. Both porins showed a slight preference for anions (Table 2).

4. Discussion

PorB/C3 porin has been overexpressed in *E. coli* and the overall structural and functional properties of this porin restored and found to be nearly identical to the native wild type counterpart (Ref. [1] and unpublished data). Previous work showed that both recombinant and wild type porins exhibited a high content of β-sheets and existed as unit trimers [2]. The trimeric porin is tightly folded as evidenced by resistance to proteases and lack of aqueous exposure of tyrosine residues. It also formed permeability pathways impermeant to stachyose, with an estimated pore size of 1.6 nm, the same as the wild-type channel (Ref. [2] and unpublished data). In the present studies, very minor differences between the recombinant and wild-type channels could be demonstrated through very careful, detailed, electrophysiological investigation. While statistically significant, only the selectivity change may have physiological significance. The underlying reason for the difference in the selectivity of the recombinant PorB/C3 is unknown. Is it due to an alternative folding pathway or the presence in the wild-type of some tightly bound lipid? Further investigation will be needed to resolve this issue.

The detailed analysis of these channels has given new insight into their function. Most important is the fact that these channels actually gate at very low potentials and we calculate that even at 0 mV only 60% of the channels should be open (given enough time to adjust to the experimental conditions). Previous reports of the electrophysiological properties of porins used step voltages for a fixed length of time to generate conductance/voltage plots. The voltage dependence of the rate of closure means that at low voltages, the rates are exceedingly slow. Thus, within the observation time, the channels did not reach equilibrium. By using slow triangular voltage waves and focusing on the rapid opening process, we have reached a reasonable level of equilibrium with the easily accessible closed states. The reason for this finding relies on the fact that when we reduced the frequency of these waves from 5 to 2 mHz, we found no significant change in the voltage-dependence parameters obtained from the opening process (data not shown). The closing process did become more complete at the lower frequency but the properties of the opening process did not change significantly.

Another important finding was that the inherent asymmetry changes from membrane to membrane. This asymmetry was reported by others [7,8] in the related porin from *N. gonorrhoeae*. However, if they noticed the variation from membrane to membrane, they seem not to have realized its potential significance. It is clear from examining single channels (data not shown) that closure occurs at lower potentials for one sign of the potential just as it does for multi-channel membranes. Thus, the asymmetry seems inherent in each channel and is not a property of channel–channel interactions. These channels should be (from analogy to other porins) inherently structurally asymmetric and this probably underlies their functional asymmetry. The fact that this asymmetry is still seen in a multi-channel membrane means that the channels are probably inserting into the membrane in the same direction. The fact that this direction varies from membrane to membrane would mean that the channels are interacting cooperatively during the insertion process. Such behavior was reported for the mitochondrial channel, VDAC [29]. For VDAC, this behavior has been shown to be due to VDAC's ability to catalyze the insertion of uninserted VDAC channels (increases the rate of insertion by a factor of 10^{10}) and thus the first channel that inserts determines the direction of subsequent insertions. This property has been named 'auto-directed insertion'. From the observations on PorB/C3, it seems that this channel may have this same property, which is also observed in the recombinant protein.

The recovery of native structure and function of membrane proteins extracted from inclusion bodies is essential to the usefulness of the recombinant approach to produce large quantities of membrane proteins. The data from our studies further confirm that of others [1,2] and add evidence that the refolding procedure employed with the recombinant porins restore all the known functions as well as structure to the PorB/C3 of *N. meningitidis*. The reported differences are small and perhaps are inconsequential but

may indicate some as yet not understood aspect of this porin.

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