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# Channel-closing activity of porins from *Escherichia coli* in bilayer lipid membranes

Guangzhou Xu [a], Biao Shi [b], Estelle J. McGroarty [a] and H. Ti Tien [b]

[a] *Department of Biochemistry and* [b] *Department of Physiology, Michigan State University, East Lansing, MI 48824 (U.S.A.)*

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**The opening and closing of the ompF porin from *Escherichia coli* JF 701 was investigated by reconstituting the purified protein into planar bilayer membranes. The electrical conductance changes across the membranes at constant potential were used to analyze the size and aggregate nature of the porin channel complexes and the relative number of opening and closing events. We found that, when measured at pH 5.5, the channel conductance diminished and the number of closing events increased when the voltage was greater than 100 mV. The results suggest that the number of smaller sized conductance channels increases above this potential. There was also an increase in the smaller subunits and in the closing events when the pH was lowered to 3.5, and these changes were further enhanced by increasing the voltage. We propose that both lowering the pH and elevating the potential across the membrane stabilize the porin in a conformation in which the subunits are less tightly associated and the subunits open in a non-cooperative manner. These same conditions also appear to stabilize the closed state of the pore.**

## Introduction

The porin proteins in the outer membranes of mitochondria and Gram-negative bacteria have been studied extensively in recent years [1]. These porins, which are thought to have evolved from a common ancestral protein, function as molecular sieves, allowing small hydrophilic molecules to pass across the membrane. The best characterized porin is the ompF protein of *Escherichia coli*. The primary sequence of this protein is known [2]. Its secondary structure, as revealed by circular dichroism of detergent solubilized protein [3] and X-ray diffraction analysis of crystallized protein [4,5], is high in $\beta$-structure.

Electron micrographs of bacterial porin [6,7] and conductance measurements across bilayer membranes containing porin suggest that, in the membrane, the protein exists as trimeric units of three identical polypeptides [8,9]. However, information on the tertiary and quaternary structure of these porin aggregates and conformational changes that occur in these complexes is still far from complete. Analysis of the structure of the ompF trimer in the electron microscope has indicated that three openings exist on the outer face of the complex. These channels appeared to fuse in the middle of the membrane, forming a single channel exiting on the inner face of the membrane [7]. Such a branched channel model would predict that the porin subunits, if separated from the other subunits, would be unable to form a chan-

Abbreviations: SDS, sodium dodecyl sulfate; CIE, current increment events; CDE, current decrement events; PC, phosphatidylcholine; PE, phosphatidylethanolamine.

Correspondence: Dr. E.J. McGroarty, Department of Biochemistry, Michigan State University, East Lansing, MI 48824, U.S.A.

nel. Furthermore, in such a complex, the central constriction would be limiting for the movement of ions [1]. Closing of only one of the three external portals would decrease the current across the pore by only about 5% and closing a second external entrance would decrease the current by another 10–20% [1]. In contrast, if each of the porin subunits within the trimer contained a complete and separate channel through the membrane, closing one or two of the subunits would decrease the conductance by 33% or 66%, respectively, of that for the entire complex. In this study, we detected conductance changes of the ompF protein in a bilayer membrane of 33% and 66% of that of the main channel event. Our results suggest that units containing three channels are the major conductance channel, but monomers and dimers had channel-forming activity and that these smaller conductance channels increase under conditions where the interactions between the subunits within the trimer were weakened. In the present study, we have also analyzed voltage controlled properties of bacterial porin channels. The size of the porin channels of the mitochondrial outer membrane has been shown to be voltage-dependent; that is, the size of the single-channel conductance decreases and the number of closing events increases with increasing voltage [10–12]. Also, Schindler and Rosenbusch [8,13] found that bacterial porins inserted in a bilayer lipid membrane close when the electrical potential exceeds a threshold value of about 140 mV. Others [14,15], however, have been unable to show such effects and some investigators have asserted that the small number of channel closing events is unaffected by the sign or size of the membrane potential [16].

In this study, we analyzed conductance across bilayer lipid membranes containing ompF porin. We measured more than $10^3$ discrete electrical current increment events (CIE), presumably reflecting channel opening, and current decrement events (CDE), representing channel closing. We found that the size distribution of the channels and the percent of total events which were CIEs decreased with increasing membrane potential.

## Material and Methods

*Porin preparation*

The ompF protein was isolated and purified from *E. coli* strain JF 701 [17]. The bacteria were grown and the protein-lipopolysaccharide-peptidoglycan complex was isolated as described by others [18]. The isolation and purification of the protein was similar to that used by Benz and co-workers [14] with some modification. The trypsin treatment was repeated four times to remove the peptidoglycan layer. A CL-Sepharose-4B column was used for the fractionation of the protein aggregates. The protein content in the eluted solution was monitored by measuring absorption at 280 nm. The main protein-containing peak eluted as an aggregate of about 800 kDa [19] and was collected and stored at $-20°C$. The isolate produced a single band on sodium dodecyl sulfate (SDS)-polyacrylamide gels. Before addition to the membrane system, the sample was solubilized in solution A, comprised of 0.1% SDS, 3 mM $NaN_3$, 5 mM Tris-HCl (pH 8) and frozen and thawed multiple times ($> 20$). Between 1 and 5 $\mu$l of porin in solution A at 200 $\mu$g/ml was added to a 7 ml solution of 0.1 M NaCl or of 0.1 M KCl (pH 3.5 or pH 5.5) for 30 to 120 minutes prior to analysis in a bilayer lipid membrane.

*Bilayer lipid membrane*

The principle and techniques used to study bilayer lipid membranes have been described previously [20]. The lipids used to form the membrane included oxidized cholesterol, soybean phosphatidylcholine (PC, Sigma Chemical Co.), and L-$\alpha$-phosphatidylethanolamine (PE, type V, from *E. coli*, Sigma Chemical Co.). Oxidation of cholesterol (Eastman Chemicals) was performed by the method of Tien et al. [21]. The membrane was formed by applying a 1.5% lipid solution in *n*-decane to a hole in a teflon chamber submersed in a bathing solution at pH 5.5 or 3.5. The lifetime of the bilayer lipid membranes was $> 0.5$ h and the resistance was $> 10^8\ \Omega \cdot cm^2$ in the absence of porin. The pH was adjusted using HCl and measured with a pH meter. The membrane was monitored with a microscope until it turned black, indicating that a bimolecular leaflet had formed. Porin was added either before or after the membrane became black. Constant voltage was maintained during all experiments, and the current across the membrane was measured using a Keithley model 610 electrometer.

The stepwise conductance changes, $\Lambda$, across the membrane were measured and divided by the specific conductance, $\sigma$, of the solution. Assuming that the porin channel is a hollow water-filled cylinder, then $\Lambda/\sigma = \pi r^2/l$ where $r$ is the inner radius of the channel at its narrowest section and $l$ is the channel length. In this study, most conductance changes are reported as the size parameter $\Lambda/\sigma$ since this parameter is proportional to the cross-sectional area of the channel and corrects for the changes in conductances at different pH values and in different salt solutions.

## Results

Abrupt stepwise increases and decreases in the current across the bilayer lipid membrane in the presence of porin were recorded from the electrometer, as shown in Fig. 1. We assume that the CIE represent opening of porin channels, while the CDE represent closing events (arrows, Fig. 1). The size parameter, $\Lambda/\sigma$, represents the conductance change of the membrane ($\Lambda$) divided by the specific conductance of the bathing solution ($\sigma$) and is presumed to be proportional to the cross sectional area of the channel at its narrowest point (see Material and Methods). At low voltages (< 100 mV), the majority of conductance changes had size parameter values of approx. 3.1 Å, while a few smaller current jumps, indicated by M (Fig. 1), were detected which had size values approximately one-third of the main conductance change.

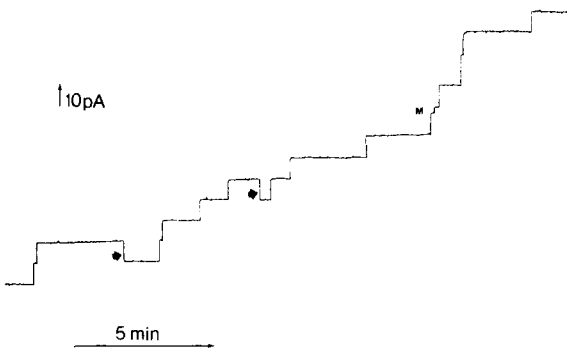


Fig. 1. Stepwise current changes across a membrane comprised of PC/oxidized cholesterol (2:1) in the presence of 60 ng/ml of ompF protein in a bathing solution of 0.1 M NaCl (pH 5.5). The porin was pretreated by repeated freezing and thawing in solution A, followed by preincubation in the bathing solution for 30 to 60 min at room temperature. The current decrements, indicated by arrows, presumably represent channel closing events of porin trimers and have the same size as the majority of increment events. The current change, indicated by M, has a magnitude one-third of the majority of events and is assumed to indicate the opening of a single channel. The voltage across the membrane was 50 mV. The baseline of the tracing was omitted.

The size parameters of both CIE and CDE were recorded at different transmembrane potentials. In these studies the bilayer lipid membrane, comprised of a 2:1 mixture of PC/oxidized cholesterol (by weight), was bathed in 0.1 M NaCl (pH 5.5), and the transmembrane potential maintained at values between 25 and 150 mV. At each voltage, approximately 200 individual events were measured, and histograms of the relative number ($P$) of CIE and CDE events were plotted against the $\Lambda/\sigma$ values (Fig. 2). One can see that at all voltages studied, the main channel size was approx. 3 Å (Table I). However, with increasing voltage, smaller channel events became prominent (Fig. 2, Table I). At a transmembrane potential of 125 mV, three distinct populations of channels were evident: the main channel of size 3.1 Å, and two smaller channels, 0.9 and 1.7 Å in size. These smaller channels have cross-sectional areas approximately one-third and two-thirds the size of the main conductance channel. Above 75 mV, the number of large conductance jumps ($\Lambda/\sigma > 4$ Å) increased, which may reflect protein aggregation

TABLE I

SIZE ($\Lambda/\sigma$) OF SINGLE-CHANNEL EVENTS AT pH 5.5 FOR ompF MEASURED AT DIFFERENT MEMBRANE POTENTIALS

| Voltage (mV) | CIE+CDE | | CDE |
|---|---|---|---|
| | Main peak (Å) | $\overline{\Lambda/\sigma}$ [a] (Å) | $\overline{\Lambda/\sigma}$ [b] (Å) |
| 25 | 3.1 | 2.9 | n.d. [c] |
| 50 | 3.1 | 2.9 | n.d. |
| 75 | 3.1 | 2.7 | n.d. |
| 100 | 3.0 | 2.9 | 2.6 |
| 125 | 3.1 | 2.4 | 2.1 |

[a] Average size of all CIE and CDE smaller than 4 Å, as shown in Fig. 2.

[b] Average size of all CDE as shown in Fig. 3.

[c] n.d., not determined.

induced by the high potentials. Also, the uniformity in the size of the conductance channels decreased at the high potentials, as has been reported in studies of mitochondrial porin [11].

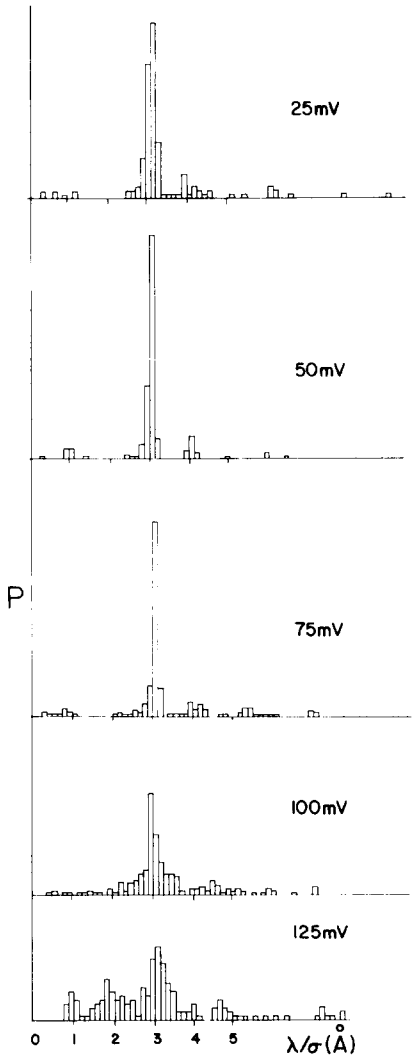


Fig. 2. Distribution of the size parameter, $\Lambda/\sigma$, for ompF protein added to a bilayer lipid membrane comprised of PC/oxidized cholesterol (2:1) at different transmembrane potentials. $\Lambda$ is the conductance change and $\sigma$ is the specific conductance of the bathing solution. $P$, in arbitrary units, is the relative number of events with the give size parameter values. The experimental conditions were the same as that described for Fig. 1, except that the porin used in the top histogram (25 mV) was added at a concentration of 30 ng/ml, and this protein was added to the bilayer lipid membranes without pretreatment. Both the opening and closing events are included in the histograms.

From the experiments described in Fig. 2, the CDE measurements alone were plotted as separate histograms and are shown in Fig. 3. The number of CDE was relatively small when the transmembrane potential was under 75 mV, but increased above this voltage. At 100 and 125 mV, the average sizes of the CDEs were significantly smaller than the size of the total events (Table I). Furthermore, the average size of the CDE shifted to smaller values when the potential was raised from 100 to 125 mV (Table I).

In preliminary studies, we have found that the size distribution of the ompF porin channels was essentially identical when the bathing solution surrounding the bilayer lipid membrane was changed from 0.1 M NaCl to 0.1 M KCl (pH 5.5) (unpublished data). However, when the pH of the bathing solution was lowered to 3.5, a treatment which reportedly decreases subunit interaction within the porin trimers [16], the size distribution of porin channel conductance is dramatically altered. The size distribution histograms of ompF porin channels inserted into bilayer lipid membranes bathed in 0.1 M KCl (pH 3.5) are shown in Fig. 4. When the transmembrane potential was maintained at

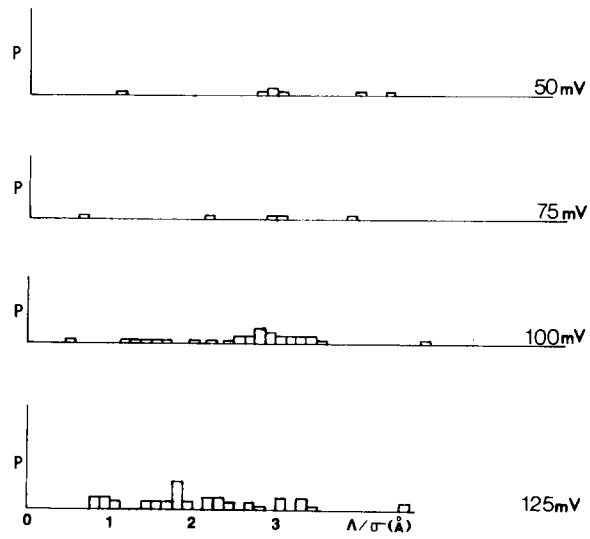


Fig. 3. The distribution of the size parameter of only the closing events from Fig. 2 at different transmembrane potentials. The experimental conditions were the same as for Fig. 2.

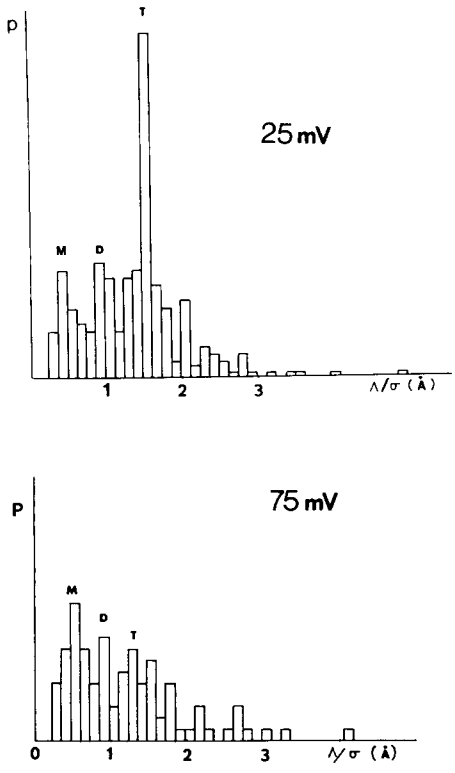


Fig. 4. Distribution of the size parameter, $\Lambda/\sigma$, for the ompF protein in a bilayer lipid membrane comprised of PE/oxidized cholesterol (2 : 1). The porin sample, at a final concentration of 140 ng/ml, was incubated in the bathing solution of 0.1 M KCl (pH 3.5), for 0.5 to 2 h at room temperature prior to addition to the bilayer lipid membrane. 195 events measured at the two voltages were analyzed.

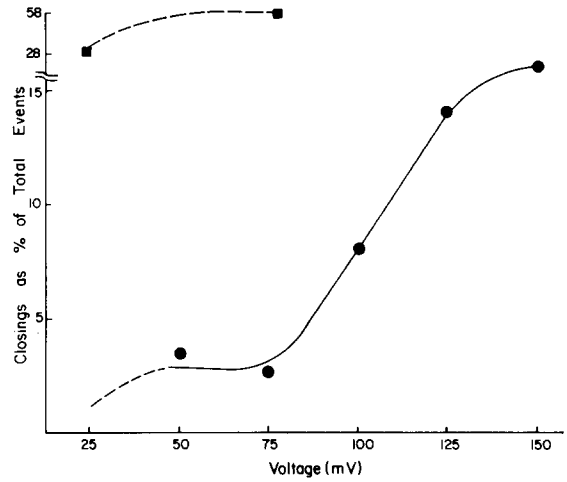


Fig. 5. Voltage dependence of porin channel closing from ompF protein suspended at pH 5.5 in 0.1 M NaCl (●) or at pH 3.5 in 0.1 M KCl (■). The bilayer lipid membrane systems used for the pH 5.5 studies were as described for Figs. 2 and 3, and for the pH 3.5 studies, as described for Fig. 4.

25 mV, the majority of channels (both CIE and CDE) had a size of 1.6 Å, while there were a significant number of channels with one-third and two-thirds the size of the major peak. Furthermore, when the voltage across the membrane was raised to 75 mV, the channel size distribution at pH 3.5 shifted to even smaller values (Fig. 4, 75 mV), and the majority of channels now had a size of 0.6 Å, approximately one-third the cross-sectional area of the channel at 25 mV.

Not only did the size of the channels decrease at low pH values, but the number of closing events also increased. Fig. 5 shows the percent of total events that were CDEs. At pH 5.5, using transmembrane potentials below 100 mV, the CDEs were less than 5% of the total events, while at pH 3.5, the CDEs were > 25% of the total, and approached 50% of the total at 75 mV. The results indicate that lowering the pH and increasing the transmembrane potential both decrease the size distribution of ompF porin channels and increase the probability of channel closing for ompF porin inserted into a bilayer lipid membrane. Furthermore, when the transmembrane potential was decreased from 125 mV to 25 mV, the number of closing events decreased and the channel size increased, indicating that changes induced at high voltage were reversible (data not shown).

## Discussion

The changes in conductance across a bilayer lipid membrane containing porin protein are of two basic types; increases in conductance (CIE) and decreases in conductance (CDE). In most bilayer lipid membrane studies of porins the majority of events have been reported to be CIE and are interpreted to represent the opening of porin channels [8,13]. Others have noted infrequent CDE and suggest that, under the conditions used in bilayer lipid membrane studies, porin channels close relatively infrequently [16].

In our studies, we have noted two types of CDE. The vast majority of CDE occur very fast ($< 0.3$ ms) and are of a magnitude essentially identical to the CIE. On rare occasions at low pH or in high ionic strength, we detected very slow closing events which occurred over a period of seconds to minutes. These slow events showed random fluctuations in the conductance during this time. We propose that the fast CDE represent a reversible conformational change in the porin proteins from an 'open' to a 'closed' state. The slow process seen on rare occasion is thought to reflect an irreversible denaturation of an open porin channel.

In analyzing both the CDE and CIE at pH 5.5, the peak value of the channel size at all voltages was 3.1 Å. Assuming a channel length of 7.5 nm [22], we calculated that this size corresponds to an interior diameter of 1.7 nm, consistent with other bilayer lipid membrane results [8,16]. Below 100 mV, there was little change in the size distribution histogram of conductance changes measured at pH 5.5. Thus, the structure of the ompF complex was unaltered by these changes in the electric field. Within this range, Ohm's law has been shown to be valid for porin single-channel conductance measurements [16]. However, above 100 mV, the channel size distribution histogram changed dramatically (Fig. 2). In addition to the main channel of 3.1 Å, two additional peaks at approx. 1 and 2 Å were evident.

We propose that the main conductance change with a size parameter value of 3.1 Å represents the opening of a porin complex that contains three separate channels which open cooperatively. At high membrane potential, the cooperativity between the units within the complex appears to be weakened and the channels more readily open independently. This would account for conductance changes corresponding to channel cross-sectional areas of one-third and two thirds that of the main complex. For the single channel with a size parameter of 0.9 Å, the calculated diameter would be 0.9 nm, consistent with the size exclusion limit of the ompF porin determined by liposome swelling assays [19,23]. Our results, however, cannot determine whether, in this model, the single channel with a 0.9 nm diameter represents a protein trimer with a single fused channel or a single protein subunit. Channel conductance studies of mitochondrial porins also have shown that the size of the conductance channels decreases with increasing membrane potentials [10–12]. In these studies, distinct size populations of channels were also detected which changed in levels with membrane potential [11,12]. These results, however, were interpreted in terms of multiple conformations of the porin channel with different channel sizes. We do not believe that the three populations of channel sizes that we see for the ompF protein represent three conformations with three sizes of channels. If this were the case, the predominant conformation with a conductance size of 3.1 Å would have a diameter of 1.7 nm, much larger than the exclusion limit of the ompF protein.

In addition to the change in the size distribution of the channels, high voltage also affected the stability of the open channel conformation. At low voltage, the number of CDE was very small, and below 100 mV, the size distribution of the CDE was difficult to assess. However, above 100 mV, the stability of the closed state increased with increasing voltage (Fig. 5). The size of the CDE was distinctly smaller than that of the total events (Table I), suggesting that, for our model, the closing event shows less cooperativity between the subunits than the opening event. A similar increase in number of CDE with increasing potential has been noted for mitochondrial porins, and CDE of the mitochondrial porins are also reported to be smaller in size than the opening events [10–12].

Processes similar to those seen at high voltage were also detected at low voltage when the pH of the bathing solution was decreased to 3.5. As shown in Fig. 4, at 25 mV, channels of one-third and two-thirds the size of the main channel were detected in sizable amounts. Furthermore, when the voltage was increased to 75 mV, the monomeric channels were the major channels detected. Thus, even at low pH, increased voltage further reduced either the aggregation state or the cooperativity in the opening of the porin channels. The smaller size of the porin trimer at pH 3.5 ($\Lambda/\sigma =$ 1.6 Å) compared to pH 5.5 ($\Lambda/\sigma = 3.1$ Å) is thought to reflect a specific pH-dependent alteration in protein structure that significantly affects the apparent channel size. The change in confor-

mation that alters the diameter of the channel at low pH is probably not the cause of the increase in channel closing events. High voltage caused these later changes, but did not affect the size of the main channel event. Furthermore, increasing the voltage at low pH to 75 mV further enhanced the level of monomeric units and CDE, but did not alter trimer or monomer channel size.

At pH 5.5, there appears to be a threshold potential, above which the porin subunits undergo a reversible, voltage-dependent change in conformation. This second, voltage-induced structural change triggered above 75 mV and enhanced at low pH induces two detectable changes in porin function in the bilayer lipid membrane. The first is an increase in the number of smaller subunit channels (CIE and CDE), and the second is an increase in the probability of channel closing. These two changes are likely caused by the same structural alteration in the protein. We propose that, both at low pH and at high transmembrane potential, the proteins within the trimeric unit become less tightly associated. In the loosened complexes, the separate channels open and close independent of the other subunits, and the closed conformation becomes more favorable. This model is consistent with the studies of Markovic-Housley and Garavito [24] and Schindler and Rosenbusch [3], who show that, below pH 4.5, structural changes are detected which are at least partially reversible and are dependent on the detergent used to solubilize the sample. These pH-dependent structural changes are thought to weaken inter-subunit contacts and change the porin structure [24], and thus, may decrease the cooperativity of the CIE/CDE of the subunits and increase the stability of the closed state. Furthermore, this less tightly associated complex is stabilized in a bilayer lipid membrane by elevated membrane potentials.

Whether transmembrane potentials control porin activity on the intact cell has been a controversial question. It has been calculated that the Donnan potential across the outer membrane usually does not exceed approximately 30 mV [25]. Furthermore, in studies where the Donnan potential was elevated to as high as 80 mV, the rate of cephaloridine diffusion through the porin channels was unaffected [26]. However, we found that such a potential may have been near the threshold value needed to induce an alteration in porin structure. In addition, lowering the pH may dramatically drop the threshold potential required to induce channel closing events. Thus, several environmental factors, such as pH and salt concentration, may dramatically affect porin-channel activity and allow for voltage-dependent control of diffusion across the channel. We found that we could detect distinct size populations of porin subunits and CDEs when the porin samples were pretreated by repeated freeze-thaw cycles, followed by preincubation in 0.1 M NaCl. In the absence of such pretreatment, the histograms were very broad and almost no CDEs were detected, even above 75 mV (data not shown). We believe that the pretreatment did not denature the protein, but allowed for dissociation of porin aggregates to trimeric units and perhaps weakened the interactions within the trimers.

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