***E. coli* PhoE porin has an opposite voltage-dependence to the homologous OmpF**

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We used patch clamp analysis to compare the electrophysiological behavior of two related porins from *Escherichia coli*, the anion-specific PhoE and the cation-selective OmpF. Outer membrane fractions were obtained from strains expressing just one of these porin types, and the channels were reconstituted into liposomes without prior purification. We show that the orientation of the reconstituted channels is not random and is the same for both PhoE and OmpF. Like cation-selective porins, PhoE shows fast and slow gating to closed levels of various amplitudes, testifying that the channels visit multiple functional states and behave as cooperative entities. The voltage-dependence of PhoE closure is asymmetric, but strikingly, occurs at voltages of inverse polarity from those promoting closures of OmpC and OmpF. Both slow kinetics and inverse voltage-dependence are removed when 70 amino acids from the N-terminal of OmpF are introduced into the homologous region of PhoE. This novel observation regarding the voltage-dependence of the two channel types, along with published results on PhoE and OmpF mutants, allows us to propose a molecular mechanism for voltage sensing and sensor charge movements in bacterial porins. It also offers new cues on the possible physiological relevance in bacteria of this common form of channel modulation.

Keywords: channel/*Escherichia coli*/porin/voltage-dependence

Introduction

The OmpF and PhoE porins are channel-forming proteins of the outer membrane of *Escherichia coli*. At the amino acid level, they share ~60% homology with each other and with another bacterial porin, OmpC (Mizuno *et al.*, 1983). They can assemble as homo- or heterotrimers (Gehring and Nikaido, 1989). Each monomer is folded as a β -barrel and contains a central pore (Cowan *et al.*, 1992). Besides permeation to cations, OmpF and OmpC allow the diffusion of larger hydrophilic solutes such as amino acids or sugars, while PhoE is specialized in the transport of anionic phosphate-bearing compounds (Nikaido, 1996).

All three general diffusion porins (OmpF, OmpC and PhoE) have been extensively studied by electrophysiology (Benz, 1988; Nikaido, 1996; Delcour, 1997). Although traditionally described in black lipid membrane as large

channels with little gating activity and mild voltage-dependence (Benz *et al.*, 1978; Schindler and Rosenbusch, 1978; Lakey *et al.*, 1985; Dargent *et al.*, 1986; Morgan *et al.*, 1990), both OmpF and PhoE display more complex kinetic and pore properties when the purified proteins are investigated with the patch clamp technique. Most notably, they show fast and slow gating kinetics, testifying that the channels exist under multiple conformational states (Berrier *et al.*, 1992, 1997). This complex behavior was also documented earlier for OmpC and OmpF when outer membrane fractions were reconstituted into liposomes and subjected to patch clamp analysis (Delcour *et al.*, 1989, 1991; Delcour, 1997).

Even when investigated with the same electrophysiological technique, the voltage-sensitivity and pore properties of porins are dependent on the nature of the biological material (Lakey and Pattus, 1989; Saxena *et al.*, 1989; Buehler *et al.*, 1991). For our reconstitution of porins into giant liposomes, we have chosen as a channel source outer membrane fractions made from strains expressing a single porin type. From our extensive patch clamp studies of OmpC and OmpF and their mutants, in this preparation and in native systems (Delcour *et al.*, 1989, 1991; Buechner *et al.*, 1990; Delcour, 1997; Iyer and Delcour, 1997; Liu *et al.*, 1997), we have ascertained that the recorded activities originate from these porins and that the channels reconstitute exclusively with the *in vivo* orientation. The channels remain highly clustered and are likely to still be surrounded by some of the native lipids, including lipopolysaccharides. In this system, we have found the conductance of the channels to be smaller, and the voltage-dependence to be more pronounced, than when porins are first purified and then studied in black lipid membranes or liposomes.

We present here a direct comparison of the voltage-dependence and gating properties between OmpF and PhoE. Both proteins can visit multiple functional states with fast and slow kinetics, as shown previously (Berrier *et al.*, 1992, 1997). The most striking difference between the two channel types is their opposite voltage-dependence. This significant distinction was not reported in previous studies, either because porins showed a symmetric voltage-dependence (Schindler and Rosenbusch, 1978; Dargent *et al.*, 1986; Lakey and Pattus, 1989), or because, when some form of asymmetric behavior was found, the asymmetry appeared to be the same for PhoE and the cationic porins (Morgan *et al.*, 1990; Berrier *et al.*, 1992, 1997). In these studies, porins were purified before electrophysiological investigation. We believe that in our system, the preservation of some of the natural membrane environment around the porins allows the channels to reconstitute in a highly asymmetric fashion and to conserve some of their natural properties. This distinct sensitivity of OmpF and PhoE to voltages of opposite polarities has allowed us to

postulate a model for the movement of sensor charges within the channel protein, and to make suggestions regarding the fundamental question of the physiological relevance of voltage-dependence in porins.

Results

Electrophysiological signatures

To study the electrophysiological properties of PhoE, we chose an OmpR strain that does not express any OmpC and OmpF porins (confirmed by gel electrophoresis; data not shown), and constitutively produces PhoE porin from a plasmid-borne gene. We obtained outer membrane fractions enriched in PhoE from such a strain and reconstituted them into giant liposomes suitable for patch clamp. Purification and reconstitution procedures can drastically affect porin behavior (Lakey and Pattus, 1989; Saxena *et al.*, 1989; Buehler *et al.*, 1991). Therefore, in order to minimize manipulations and reproduce conditions that are closer to the natural ones, we deliberately did not purify the PhoE porin. The importance of lipopolysaccharides (LPS) for channel function has been documented (Schindler and Rosenbusch, 1981; Buehler *et al.*, 1991). Since porins strongly associate with each other and with LPS, it is likely that by using membrane fractions we can retain a proteinic and membrane environment around the channels that is closer to the natural one than if porins had been first purified. Since by the use of this approach, non-porin proteins are also present in the patches, we ascertained that the recorded activities are truly from porins by the use of bacterial genetics. We are able to recognize the activities of OmpF and OmpC porins because they are absent in null mutants and modified in point mutants of the specific gene (Delcour *et al.*, 1991; Liu *et al.*, 1997). Similarly here, we have ascertained that the recorded activity is derived from the PhoE protein from the following observations: (i) the activity of this channel is absent in an isogenic strain that does not express any PhoE (because it has lost the plasmid) and no other channel activities are observed (see Figure 4); (ii) the activity is modified in an isogenic strain that does not express PhoE but a hybrid of PhoE and OmpF (see Figure 5).

As do OmpC and OmpF, reconstituted PhoE channels cluster in patches and display a high open probability at low transmembrane potentials. These two features result in a large current through porin-containing patches which is arbitrarily chosen as a baseline (labeled 'BL' in Figure 1). Since each porin monomer contains an ion-conducting pore and the patch clamp technique provides a high time resolution, it is possible to detect the current flowing through single monomers (see further discussion below). The channels display at least two kinetically distinct closed states: a long-lived state that is often attained by several monomers in a cooperative fashion and can last for several seconds (Figure 1), and a short-lived state that involves any number of monomers and is best illustrated by the brief closures of the expanded trace (see Figure 4A). In Figure 1, we have indicated the current levels reached after closures of 6–36 monomers by tick marks labeled C6 to C36. The stability of the long-lived closed state has a clear asymmetric voltage-dependence; prolonged closures of multiple channels are lengthier, occur more frequently, and develop sooner after a voltage step to

Fig. 1. Kinetic signatures of PhoE and OmpF channels. Representative current traces were obtained from a patch containing PhoE or OmpF, at the indicated pipette voltages. The preferred current level is denoted 'BL' (base line). Current levels reached after closures of 6–36 monomers are indicated by tick marks labeled C6 to C36.

positive pipette potentials than negative ones (Figure 1). A similar asymmetry has been reported by Berrier *et al.* (1997), but at potentials of somewhat larger magnitude than here. These differences may be due to the fact that porins are not purified in this study.

The voltage-dependence and kinetic signature of PhoE are very distinct from those of OmpF. Second-long closures of OmpF are much less frequent, especially in the voltage range of -100 to $+100$ mV. Apparent inactivation of OmpF channels does occur, but is somewhat variable from patch to patch. The voltage-dependence in OmpF is manifested by an increase in the frequency and the duration of fast closures. Strikingly, the asymmetric voltage-dependence of OmpF is of opposite sign to that of PhoE. OmpF channels tend to close when negative pipette potentials are applied to the patch (Figure 1). From the asymmetric voltage-dependence that we have previously documented for porins *in vivo* (Buechner *et al.*, 1990), we know that OmpF and OmpC channels reconstitute asymmetrically such that the extracellular side faces the pipette. Hence negative pipette potentials correspond to positive membrane potentials.

A comparison of the asymmetric and opposite voltage-dependencies of OmpF and PhoE is shown in Figure 2. We deliberately chose not to represent NPo (open probability), because its calculation requires the current value corresponding to all channels of the patch being closed. This

Fig. 2. PhoE and OmpF have an opposite voltage-dependence. The total number of closures was measured from 20 s recordings taken from the same experiments as in Figure 1. The values obtained for OmpF (closed symbols) and PhoE (open symbols) are plotted according to the left and right ordinates, respectively.

value is unknown, and may be variable from patch to patch and with membrane potentials. Instead, the higher propensity of channels to close as a function of membrane potential is represented as an increase in the total number of closures in 20 s. The main point of the figure is that an increase in the frequency of closures is observed at negative pipette potentials for OmpF, but at positive ones for PhoE. The sensitivity of PhoE to voltage appears greater than in a previous report (Berrier *et al.*, 1997), because a substantial increase in gating activity is already observed at +70 mV. A similar trend was observed for PhoE in 36 patches from six membrane preparations, and for OmpF in eight patches from two preparations in this study and numerous previous experiments (Delcour *et al.*, 1989; Delcour, 1997).

Reconstituted PhoE and OmpC porins have the same orientation

There are two possible explanations for the opposite voltage-dependence of PhoE and OmpF (or OmpC): (i) the channels reconstitute in the same way but have different intrinsic voltage-dependence, or (ii) the channels have the same intrinsic voltage-dependence but prefer to reconstitute in opposite orientations. To distinguish between the two scenarios, we have investigated the voltage-dependence of porins in outer membrane fractions isolated from a strain that expresses both OmpC and PhoE, and that was readily available in our laboratory. It is known that when both porin types are expressed, a mixture of homo- and heterotrimers exists in the membrane (Gehring and Nikaido, 1989). Assuming that monomers assemble randomly into trimers, the relative proportions of different trimers should be 25% of OmpC homotrimers, 25% of PhoE homotrimers and 50% of heterotrimers.

Figure 3A and B shows a cartoon of the electrophysiological signature expected from a population of co-expressed OmpC and PhoE channels that behave according to the two scenarios outlined above. According to the first scenario (both porin types reconstitute with the same orientation), the patches should contain four types of trimer, as shown (Figure 3A); only two types of heterotrimer (CCE and EEC) are expected because of the circular arrangement of monomers. The current traces expected for each type of trimer are drawn, with downward deflections

representing closures at negative pipette voltages (–) and upward ones representing closures at positive pipette voltages (+). We assume that the voltage-dependence of individual channel monomers is not drastically affected in the heterotrimeric form, and that one should still be able to observe the characteristic gating pattern of each porin type at the appropriate voltages: long closures of PhoE channels and low levels of fast closing kinetics of OmpC at positive pipette potentials; no PhoE long closures and only fast OmpC closing kinetics (with an increased frequency compared with positive pipette potentials) at negative potentials. The bottom traces represent the sum (Σ) of the activities originating from all types of trimer, and thus the expected signature to be observed experimentally. Note that the summed traces still show long closures only at positive voltages.

According to the second scenario (the proteins have an identical intrinsic voltage-dependence but prefer to reconstitute in opposite ways), we can anticipate six types of trimer, as shown (Figure 3B). Note the opposite orientation of the OmpC and PhoE homotrimers (as shown by the position of the wedge). Four types of heterotrimer would be expected: CCE and EEC have the same orientation as OmpC homotrimers, and C*C*E* and E*E*C* have the orientation of the PhoE homotrimers. This time, heterotrimers will display the characteristic PhoE long closures and fast OmpC gating at the same potentials, i.e. negative ones for CCE/EEC and positive ones for C*C*E*/E*E*C*. Now the summed traces display a mixture of long and spiky closures at positive and negative voltages, a signature that is clearly distinct from that anticipated with the first scenario.

The experimental traces from patches containing a mixture of homo- and heterotrimers of OmpC and PhoE (OmpC/PhoE co-expression) clearly demonstrate a segregation of the short and long closures at opposite voltages (Figure 3C). At negative pipette potentials, there are no long closures, but only the spiky openings and frequent transient closures typical of OmpC. At positive potentials, the characteristic PhoE long closures appear, and the fast closing kinetics are reduced. These activity patterns are similar to those of Figure 3A, and are consistent with a model whereby both OmpC and PhoE reconstitute with the same asymmetric orientation, and intrinsically display opposite voltage-dependence. These results were obtained in six independent experiments from two preparations of outer membrane.

Conductance measurements

The traces of Figure 1 best illustrate the slow and occasional closures of many PhoE channels. A close inspection of expanded traces reveals upward deflections from the baseline that have a small amplitude (Figure 4A). Since each PhoE monomer has its own pore, our working hypothesis is that such events correspond to the closures of single monomers (see below). In some patches, the average time of closure of a single monomer is long enough to appear as a well-defined square-top event (bottom trace of Figure 4A). In other patches, the kinetics of closures of single monomers are much faster, and many of these fast events are too brief to be resolved. However, even when we exclude these time resolution artefacts by rejecting events lasting less than 300 μ s, the kinetic

Fig. 3. PhoE and OmpC reconstitute with the same orientation. (A) and (B) Cartoons representing the expected kinetic signature of patches containing co-expressed PhoE and OmpC. The bath and pipette sides of the membrane are shown as 'B' and 'P', respectively. Pipette voltages are negative (-) or positive (+). (C) Representative current traces obtained at the indicated pipette voltages from patches containing either PhoE or OmpC homotrimers or a mixture of homo- and heterotrimers of these two proteins (labeled 'OmpC/PhoE Co-expression'). The preferred current level is denoted 'BL' (base line). Current levels reached after closures of 6 or 12 monomers are indicated by tick marks labeled C6 and C12.

analysis still reveals the existence of a large number of monomeric transitions with an average duration of the order of 0.5–1.0 ms. The existence of two putative gating modes has also been reported by others (Berrier *et al.*, 1997).

The apparent noise of the baseline level in the PhoE+ strain is markedly increased as compared with the strain lacking PhoE, testifying that it does not represent the inherent noise of the recording system, but the rapid gating of channels between open and closed states. On occasion, as in OmpC and OmpF (Delcours, 1997), larger downward spikes are observed from the baseline, indicating that openings of additional channels do occur, albeit extremely transiently.

Because the closures of small amplitude are usually so fast, they do not appear as well-defined peaks on amplitude histograms. Thus their height has been measured from events that showed a typical square-top shape. Just as for OmpC and OmpF, such heights typically cluster around values that are roughly integer multiples of each other (see Materials and methods). In Figure 4B, we have plotted the averages and standard deviations of such clusters for several voltages in the same patch. For this experiment, the fitted lines gave conductance values of 27, 52, 77 and 110 pS. Complete current-voltage plots were generated for three experiments, and the averaged conductance values were: 25 ± 3 , 48 ± 3 , 71 ± 7 and 110 ± 2 pS.

Is the smallest conductance value (25 pS) that of a monomer, or does it represent a substate? This is a central issue in porin electrophysiology, where measurement of the 'single channel conductance' continues to be a point of divergence between various laboratories. Berrier *et al.* (1997) reported multiple conductance levels that vary from patch to patch and that are not always integer multiples of each other. They suggested the existence of several substates, but did not propose a value for the full conductance of a PhoE monomer, presumably because no single conductance value was observed consistently. In the 16 patches from which amplitude measurements were made, we have not found such behavior, although there is a distribution of individual event heights as demonstrated by the error bars of Figure 4B. However, some of the conductances that we have measured (48, 71 and 110 pS) are close to the 50, 85 and 115 pS values reported by Berrier *et al.* (1997).

We propose that the conductance values larger than 25 pS originate from multiple monomers that gate cooperatively. We favor this interpretation over that of substates because substates are usually encountered less frequently than the full-conductance state and do not appear unless accompanied by it. In our experiments, we believe that 25 pS is the monomeric conductance for the following reasons: (i) it is the size of the most frequent transition; (ii) there is no favored larger transition that accompanies it consistently and at all voltages (for example in Figure

Fig. 4. PhoE has a small conductance. (A) Current traces obtained from CE1265 strains lacking or containing a PhoE-expressing plasmid. Note the absence of any channel activity in the top trace; even though OmpR mutants are slightly leaky, the expression of OmpC or OmpF is so drastically reduced that we never encountered their activity in six patches in the PhoE-deficient strain, and 36 patches in the PhoE⁺ strain. The time scale is expanded compared with Figure 1. The baseline level is marked 'BL', and closures of one or six monomers are denoted C1 and C6. Note the different kinetics of closure of one monomer in the middle and bottom traces. The pipette voltage was +90 mV. (B) Current-voltage relationship obtained from a single patch containing PhoE channels. Each symbol is the average of 3–12 values ($\pm$ SD) obtained from closing transitions involving either 1 ($\bullet$), 2 ($\blacktriangle$), 3 ($\blacksquare$) or 4 ($\blacklozenge$) monomers. The solid lines are the best fits through the points, and the error bars sometimes lie within the thickness of the symbols.

1, a dominant large conductance is nine times the smallest event at -110 mV, but multiples of six at $+110$ mV; (iii) it is the largest common denominator of all the conductance values measured in a single patch. According to this view, the results of Berrier *et al.* (1997) could be explained by considering some variability in the extent of cooperativity from patch to patch, which we have also seen in our experiments. In addition, the discrepancy between their results and ours could stem from the use of purified porins which, upon reconstitution, may not regain a cooperative behavior identical to that found in outer membrane fractions. To test this hypothesis, purified and native porins should be tested side by side.

With a single monomer conductance of 25 pS, the slow transitions of large amplitudes observed at positive potentials correspond to the cooperative closures of six monomers. OmpC and OmpF also show cooperativity that extends beyond trimers (Delcour *et al.*, 1989; Buechner *et al.*, 1990; Delcour, 1997; Iyer and Delcour, 1997). If the monomeric conductance were 50 pS, we could consider these large transitions as closures of trimers, but the 50 pS

level is rarely encountered, and thus is unlikely to represent the full-conductance state.

Finally, experiments in asymmetric conditions showed that the recorded activity is that of an anion-selective channel, as expected (data not shown).

OmpF-like properties of a PhoE/OmpF chimera

In order to gain some insight into the molecular basis for the asymmetric voltage-dependence of OmpF and PhoE, we have studied the electrophysiological behavior of a PhoE/OmpF chimera. This hybrid polypeptide is most of the PhoE sequence with residues #3 to #73 replaced by the homologous amino acids of OmpF (Tomassen *et al.*, 1984). This stretch corresponds to the first three transmembrane segments, the extracellular loop L1 and part of the extracellular loop L2 (but not L3). This protein was expressed from a plasmid-borne gene in the same OmpR strain as above but in the absence of PhoE. Although limited to one hybrid, this study provides compelling results that allow us to formulate hypotheses regarding the molecular basis for the opposite voltage-dependence between OmpF and PhoE.

Striking differences from the PhoE activity are shown in the traces of this hybrid channel at positive and negative pipette potentials (Figure 5A): (i) the lack of long closures at positive pipette potentials, (ii) the increased closing activity at negative voltages, and (iii) the increased frequency of openings from the baseline. All these properties are also found in OmpF channels.

The number of closing events in 35 s of recording are compared between PhoE, OmpF and this hybrid (Figure 5). Similar values have been obtained in several analyzed experiments (four for PhoE and four for the hybrid) from at least two membrane preparations for each porin type. The overall pattern of PhoE and hybrid activity was reproducible in 36 and 13 experiments, respectively. The behavior of OmpF was typical, as observed in a large number (> 80) of experiments (for example, see Iyer and Delcour, 1997). The abscissa of these graphs show the number of monomers that are simultaneously closed. Unless the closure of a specific number of monomers is favored, a monotonous decrease in the number of observed closures is expected as the number of closed monomers becomes larger. This trend is observed in OmpF and the hybrid (Figure 5C and D). PhoE, on the contrary, shows peaks at 5 and 6, 11 and 12, and 17 and 18 channels, at a positive pipette potential (Figure 5B). These peaks originate from the long closures during which 1 of 6, or of 12, or of 18 monomers oscillate between their open and closed states. Like OmpF, but unlike PhoE, the hybrid shows a greater number of closures at negative pipette potentials (open symbols) than positive ones (closed symbols). Thus, the replacement of 70 amino acids in PhoE by the equivalent OmpF sequence was sufficient to alter the voltage-dependence and the gating kinetics of the channel.

Discussion

The electrophysiological behavior of PhoE in patch clamp and black lipid membrane experiments has been described (Benz *et al.*, 1978, 1985; Dargent *et al.*, 1986; Berrier *et al.*, 1997). The major difference between these studies

Fig. 5. The PhoE/OmpF hybrid resembles OmpF. (A) Representative current traces are displayed at the indicated pipette voltages. The baseline level is marked 'BL', and the direction of channel closure (C) is indicated by the arrow. Note the absence of long closures at +90 mV, and the more frequent fast closures at -90 mV. (B), (C) and (D) The total number of closures was calculated from 35 s recordings of PhoE (B), OmpF (C) and the PhoE/OmpF hybrid (D) activity, at pipette voltages of +90 mV (closed symbols) and -90 mV (open symbols). The values are plotted against the number of channel monomers simultaneously closed during these events. The lack of a symbol indicates a value of zero.

and the work presented here is that we have not purified porins, but used native outer membrane vesicles as a source of channels in our reconstitution. We believe that this difference in biological material is the reason for the existence of much more asymmetric behavior and increased sensitivity of PhoE with respect to the applied transmembrane electric field. We have found this to be the case for OmpF and OmpC as well (Delcour *et al.*, 1989; Buechner *et al.*, 1990; Delcour, 1997). The most logical explanation for this is the likely presence of LPS remaining in the vicinity of the proteins. As suggested, the presence of LPS would affect the electric field at the extracellular mouth of the channels (Karshikoff *et al.*, 1994).

Because of this strong asymmetry in the voltage-dependence of non-purified porins, we were able to demonstrate that voltages of opposite signs promote the closures of PhoE and OmpF. This observation has a profound impact on our understanding of the relationships between function and structure of channels and of the physiological role of voltage-dependence in porins. OmpF

Fig. 6. Molecular model of the constriction zone of the PhoE porin. The image was obtained from the published crystal structure (Cowan *et al.*, 1992), and the Insight II Molecular Modeling System (Biosym Technologies). The locations of key residues of PhoE are shown, with homologous OmpF residues given in parentheses. To obtain a better view of the L3 loop spanning across the pore, most of the other extracellular loops have been clipped.

and PhoE share 60% homology and are very similar in their general structural designs. One of the striking points of similarity is the presence of negative charges on the L3 loop (Asp-106 and Glu-110 in PhoE; Asp-113 and Glu-117 in OmpF) immediately opposite a cluster of positive charges in the barrel [Lys-16, Lys-18, Arg-37, Arg-75 and Arg-126 in PhoE; Lys-16, Arg-42, Arg-82 and Arg-132 in OmpF (residue 18 is Val)] (Figure 6). Karshikoff *et al.* (1994) have calculated the electrostatic potentials in OmpF and PhoE, and shown that they have a similar screw-like form and increased amplitude in the channel's constriction zone. The major difference between the two channels was found at the constriction zone and the extracellular mouth, where the potential was close to zero for OmpF but positive in PhoE. This distinction is likely responsible for the known difference in ion selectivity of the two pores (Karshikoff *et al.*, 1994).

It is possible that this difference also influences the voltage-dependence of the channels. The presence of Lys-18 in PhoE plays an important role because of its close proximity to Glu-110 at the tip of the L3 loop. The lysine to valine substitution at residue 18 in the hybrid is the most conspicuous change introduced with the OmpF sequence. The other residues potentially implicated in the charge lining of the pore and the constriction zone of PhoE are either not touched by the substitution (Asp-106 and Lys-125 of L3, Arg-75 and Arg-126 in the barrel), or remained essentially unchanged (Lys-16 and Arg-37 in the barrel) (Figure 6). Other changes, such as Arg-55 → Gln-60 or Glu-59 → Asn-64 do not implicate residues that protrude into the pore and participate in the channel's electrostatic potential (Figure 6). So our working model is that Lys-18 plays an important role in setting up the voltage-dependence. This can be tested by introducing Lys-18 into OmpF instead of Val-18.

This hypothesis is supported by the observation that a K18C substitution in PhoE has greatly increased the

critical voltage for channel closure (Van Gelder, 1996; Van Gelder *et al.*, 1997). It is striking that the substitution of positively charged residues by neutral ones in the barrel cluster has an opposite effect in PhoE than in OmpF; it decreases voltage-sensitivity in PhoE but increases it in OmpF (Saint *et al.*, 1996; Van Gelder, 1996; Van Gelder *et al.*, 1997). This distinction also applies to the negative residues of the L3 loop, but in the opposite way; the E110C substitution in PhoE decreases the critical voltage (increases the voltage sensitivity), while the D113C or E117C mutations in OmpF increase the critical voltage (decrease the voltage sensitivity) (Van Gelder, 1996; Van Gelder *et al.*, 1997). It is usually thought that a decreased voltage sensitivity results from charge neutralization of residues forming the voltage sensor. This would implicate the positive amino acids as sensors for PhoE and the negative ones as sensors in OmpF (Van Gelder, 1996; Van Gelder *et al.*, 1997).

This is somewhat surprising, but in agreement with our observation of opposite voltage-dependence in the two porins. In both cases, channel closure would be obtained following the movement of the sensor charges towards the periplasmic side of the channel in response to the appropriately applied electric field. In other words, in OmpF, the negative sensor charges of L3 would move towards the intracellular side in response to positive voltages on that side of the membrane (i.e. negative pipette potentials in our experiments). In PhoE, the movement of positive charges of the barrel towards the intracellular side would be triggered by negative potentials applied to the cell interior (corresponding to positive pipette potentials in our experiments).

This hypothesis is supported by the mutations promoting an *increased* voltage sensitivity (Saint *et al.*, 1996; Van Gelder, 1996; Van Gelder *et al.*, 1997). For example, a lower critical voltage was found in the R42C, R82C and R132P mutants in OmpF (Saint *et al.*, 1996). A possible explanation (Van Gelder, 1996; Van Gelder *et al.*, 1997) is that the movement of the negative sensor charges of L3 in response to a positive membrane potential is favored even further when the positive charges of the barrel, which act as a 'retainer', are neutralized by mutations. In PhoE, the converse is true: the movement of the positive sensor charges is not retained as much in a mutant where some of the negative charges of L3 have been neutralized (E110C mutant), and the critical voltage is lower (Van Gelder, 1996; Van Gelder *et al.*, 1997). In both cases, the distortion of the field following these charge movements might be coupled with the conformational change leading to channel closure.

The discovery of an opposite voltage-dependence in two closely related porins might shed some light on the physiological role of voltage-sensitivity. It is probably not a way to shut porins in case of wrongful insertion into the membrane (Nikaido, 1996), but rather to play the role of regulation of outer membrane permeability in specific conditions. Interestingly, both PhoE and OmpF porins are closed by voltages that would result from the influx of the solutes that they favor. A negative membrane potential would develop during the rapid influx of negatively charged compounds through PhoE. On the contrary, a positive membrane potential would result from the influx of cations through OmpF. Since it is likely that the voltage-

dependence is part of a defense mechanism, it would provide a way for the outer membrane to seal off after a large flux of solutes has occurred through the channels. This mechanism is close to a form of desensitization of the channel. A similar phenomenon is observed during the action potential: the depolarization resulting from the influx of sodium ions enhances inactivation of the sodium channels responsible for the ionic movement. Although the capacitance of the outer membrane is not known, it is anticipated that the movement of only a few ions is sufficient to produce large potential changes (Hille, 1992). So if cells find themselves in a situation where large ionic gradients occur (such as after a sudden change in environmental conditions), regulation of the outer membrane permeability might occur via such a mechanism. This intriguing hypothesis requires further demonstration, for example with outer membrane permeability assays on strains harboring voltage-insensitive porin mutants.

Materials and methods

Strains

All strains used were *E. coli* K-12 derivatives. AW738 and AW739 express only OmpF or OmpC, respectively (Ingham *et al.*, 1990). CE1265 (OmpR- PhoE-) has no detectable expression of OmpC and OmpF, and expresses PhoE from plasmid pJP29 or the OmpF/PhoE hybrid protein from plasmid pJP47 (Tomassen *et al.*, 1984). HS107 was derived from CE1265 by loosing pJP29 after multiple subcultures in the absence of chloramphenicol. HN499 expresses PhoE and OmpC in homo- and heterotrimeric forms (Gehring and Nikaido, 1989).

Chemicals

T-broth contained 1% tryptone (Difco Laboratories) and 0.5% NaCl. LB-broth was 1% tryptone, 0.5% yeast extract and 0.5% NaCl. Azolectin (phosphatidylcholine) was from Sigma, and all other chemicals were from Fisher Scientific.

Electrophysiology

Outer membrane fractions were purified by sucrose gradient centrifugation from bacterial cells grown exponentially in LB-broth (CE1265) or T-broth (other strains). Patch clamp experiments were performed according to standard techniques (Hamill *et al.*, 1981) on lipid blisters induced from artificial liposomes containing the reconstituted outer membrane fractions (Delcour *et al.*, 1989). Protein:lipid ratios of 1:1600 to 1:1800 (w:w) were typically used, yielding seals of ~0.5 to 1.0 GΩ due to the presence of multiple open channels in each patch. Experiments were performed on inside-out patches after excision by air exposure. In all figures, the voltages indicated are pipette voltages.

Experiments were done in symmetric solutions of 150 mM KCl, 5 mM Hepes, 0.1 mM K-EDTA and 0.01 mM CaCl₂ (pH 7.2). Currents were filtered at 2 kHz (Frequency Devices) and recorded with an Axopatch-1D amplifier (Axon Instruments) on VCR tapes (Instrutech). For analysis, the data were re-filtered at 1 kHz and digitized at 100 μs sampling intervals. Analysis was done with programs written in Axobasic (Axon Instruments) and developed in the laboratory.

Analysis

We arbitrarily used as a baseline (denoted as 'BL' in figures) the current flowing through the multiple open porins of the patches. Deflections from the baseline that lead to a current reduction represent closures and are labeled 'C' in the figures. Channel amplitudes were typically obtained from inspection of individual transient closures, rather than from all-point histograms (Liu *et al.*, 1997). For each experiment, a current-voltage plot is first generated with all the measured amplitudes. At each voltage, the amplitude values distribute in clusters, and the cluster of smallest value is assigned as the smallest unitary conductance. Other clusters are assigned as 2×, 3×, 4×, ... the smallest unitary conductance on the basis of their average value. Linear regression fits were then performed with Sigma Plot (Jandel) for each set of clusters, as in the typical experiment shown in Figure 4B. Kinetic analysis of closures was done with an algorithm that uses the half-amplitude criterion to classify events lasting for more than 300 μs as closures of 1, 2, ..., N channels.

The numbers and average durations of such events are computed for a defined stretch of data. A detailed description of the analysis procedures has been given elsewhere (Iyer and Delcour, 1997; Liu *et al.*, 1997).

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