The spontaneous gating activity of OmpC porin is affected by mutations of a putative hydrogen bond network or of a salt bridge between the L3 loop and the barrel

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Introduction

One of the most conspicuous features of ion channel function is the ability of these proteins rapidly to undergo conformational changes that lead to functionally distinct states. In many cases, an energy input, such as transmembrane voltage change or ligand binding, is required. In some cases, thermal energy is sufficient to support fluctuations between conformational states, and channels can be seen to oscillate spontaneously between ion-conducting and non-conducting configurations. This spontaneous type of gating is observed in porin, a bacterial ion channel whose crystal structure is known and which can be easily manipulated genetically. In an effort to understand the molecular mechanisms that underlie these spontaneous transitions between closed and open states, we have initiated electrophysiological studies of wild-type and mutant OmpC porins.

Porins are trimeric channel-forming proteins of the outer membrane of *Escherichia coli*. Each subunit contains 16 β-strands forming a transmembrane β-barrel whose pore is constricted by the third extracellular loop (L3). We investigated the effects of site-directed mutations at two critical regions of the OmpC porin: (i) the D315A mutation targets a key component of a putative hydrogen bond network linking the L3 loop to the adjacent barrel wall and (ii) the D118Q, R174Q and R92Q mutations target putative salt bridges at the root of the L3 loop. We purified the outer membrane fractions obtained from each mutant and reconstituted them in liposomes suitable for electrophysiology. Patch clamp experiments showed that the frequency of spontaneous transitions between open and closed states is increased in the D315A, D118Q and R92Q mutants but unchanged in the R174Q mutant. These transitions are not driven by transmembrane voltage changes and represent the thermal oscillations between functionally distinct conformations. The asymmetric voltage-dependent inactivation of the channels is not affected by the mutations, however, suggesting different molecular mechanisms for the spontaneous and voltage-dependent gating processes. We propose that the positioning or flexibility of the L3 loop across the pore, as governed by the putative hydrogen-bond network and a salt bridge, play a role in determining the frequency of spontaneous channel gating. 

Keywords: channel/Escherichia coli/gating/mutant/porin
The root of the L4 loop. Marked residues: D126 and R100 are on the root of the L3 loop, R168 is at the OmpF structure showing putative salt bridges as lines between the pore, most of the other extracellular loops have been clipped. (Technologies). To obtain a better view of the L3 loop spanning across the 1992) and the Insight II Molecular Modeling System (Biosym Technologies). The image was obtained from the published crystal structure (Cowan et al., 1992) and the Insight II Molecular Modeling System (Biosym Technologies). To obtain a better view of the L3 loop spanning across the pore, most of the other extracellular loops have been clipped. (B) Detail of the OmpF structure showing putative salt bridges as lines between the marked residues: D126 and R100 are on the root of the L3 loop, R168 is at the root of the L4 loop.

Wall. These results suggest that some motion of the L3 loop might underlie the spontaneous gating even in wild-type, but is restricted in the conditions used in the experiments.

Materials and methods

Chemicals and strains

E. coli K12 strains AW738 (OmpF+ OmpC−) and AW739 (OmpF− OmpC+) (Ingham et al., 1990) were used to construct the mutants. Tryptone growth medium (T-broth) contained 1% tryptone (Difco Laboratories) and 0.5% NaCl. Luria–Bertani broth (LB) was obtained from Difco Laboratories. Azolectin (phosphatidylcholine) was purchased from Sigma and all other chemicals from Fisher Scientific. Enzymes used in molecular biology protocols were purchased from either Gibco or Promega. The DNA sequencing and purification kits were obtained from Perkin-Elmer and Promega, respectively.

Mutant construction

The unique site elimination method (USE kit, Pharmacia Biotech) was used to generate the site-directed mutants in OmpC, according to a method previously used to engineer other mutants in the same protein (Liu et al., 1997). The mutation was confirmed by DNA sequencing. The mutant plasmid was introduced in a strain deleted for OmpC and OmpF from the chromosome, as previously described (Liu et al., 1997). In all the experiments, the expression of either wild-type or mutant ompC was only from the plasmid-encoded gene.

Membrane preparation and electrophysiology

The preparation of purified outer membrane fractions was done according to a published protocol (Delcour et al., 1989a). The determination of protein concentration was done by the bicinchonninic acid method (Pierce).

For electrophysiology, an aliquot of native outer membrane was mixed with sonicated phosphatidylcholine (azolectin) at a protein-to-lipid ratio of ~1:1800 (w/w) and reconstituted according to a dehydration–rehydration protocol (Delcour et al., 1989a). This procedure yields large multimellar liposomes, which are collapsed and induced to form blisters in the presence of 20 mM MgCl2 (Figure 2A). The patch-clamp technique was applied to such blisters induced from liposomes directly in the recording chamber (bath). As shown in Figure 2B, a glass pipette (opening diameter ~1–2 µm), containing an electrode immersed in a buffered ionic solution, was placed at the surface of a blister and a small patch of membrane was isolated at the tip of the pipette (excised patch mode). The membrane patches typically harbored 10–30 channel-forming subunits of porin. The pipette was then raised away from the liposome and the bath solution was thoroughly washed with new buffer of composition identical with that in the pipette, i.e. 150 mM KCl, 5 mM Hepes, 0.1 mM K-EDTA, 10 µM CaCl2, pH 7.2. In some experiments, the KCl concentration of the bath solution was lowered to 10 mM. All solutions were filtered through a 0.2 µm filter before use. A reference electrode placed in the bath solution completed the electrical circuit.

The current through individual pores was measured in real time with a patch-clamp amplifier that also serves to clamp the membrane potential to a fixed desired value (P/C Amp, V-control in Figure 2B). Throughout the paper, the voltages given refer to those of the pipette with respect to the bath. Current measurements were made using standard patch-clamp techniques (Hamill et al., 1981) with an Axopatch 1D amplifier (Axon Instruments). The pipettes had an initial resistance of...
Table I. Nomenclature of homologous residues in OmpF and OmpC

<table>
<thead>
<tr>
<th>OmpF</th>
<th>OmpC</th>
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<tr>
<td>R100</td>
<td>R92</td>
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<td>E117</td>
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<td>F118</td>
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<td>D126</td>
<td>D118</td>
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<td>R168</td>
<td>R174</td>
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<td>E296</td>
<td>D299</td>
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<td>D312</td>
<td>D315</td>
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<tr>
<td>Y313</td>
<td>Y316</td>
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10 MΩ. The data were filtered at 2 kHz with an eight-pole Bessel filter (Frequency Devices) and stored on VCR tapes (Instrutech). For data analysis, specific segments of data were re-filtered at 1 kHz and digitized at 100 µs sampling interval. Data acquisition and analysis were performed with programs written in the laboratory using Axobasic (Axon Instruments).

Data analysis

Current values were obtained by visual inspection of well resolved closing events with clearly defined square tops. Current amplitudes of single pore-forming subunits are needed for kinetic analysis: for a pipette voltage of −60 mV; we used 1.8 pA for wild-type (Iyer and Delcour, 1997; Liu et al., 1997), 1.8 pA for the D118Q mutant and 1.6 pA for the D315A mutant (corresponding to the smallest current amplitude within bursts). The half-amplitude criterion and a minimum duration of 300 µs (Liu et al., 1997) were used to define events as closures of 1, 2, ..., N channels. The total number of such events was computed for 20 s long recordings.

Results

Disruption of a putative hydrogen-bond network

Karshikoff et al. (1994) suggested that a hydrogen-bond network exists between the L3 loop and the adjacent barrel wall of OmpF (Figure 1A). A major player in this interaction is D312, whose carboxyl group would be in close proximity to the peptidyl hydrogens of E117 and F118 at the tip of L3. This residue is conserved in OmpC as D315 (see Table I for OmpF and OmpC nomenclature). Since most of our previous work on spontaneous and engineered mutants was done on OmpC (Delcour et al., 1991; Liu et al., 1997), we continued our investigations with this protein. OmpC and OmpF are highly homologous (>60% amino acid identity), especially at the level of the barrel and L3, and we used the structural information of OmpF as a guide in the design of OmpC mutants. Figure 3A shows typical experimental traces of current recorded with the OmpC wild-type and D315A mutant.

As an additional control, we also mutagenized the neighboring residue Y316 to alanine. The pattern of activity of the D315A mutant was changed considerably compared with wild-type, whereas the Y316A mutation did not affect the gating activity of the channels.

Patches typically contain a large number of predominantly open channels. Thus, in wild-type, the current trace dwells at a favored level that corresponds to the current flowing through many open pores (about 30 in the patch on wild-type channels shown in Figure 3A). We refer to this preferred level as the baseline (BL). Upward deflections from this baseline represent the transient, often cooperative closures of the predominantly open channels. This type of activity is not the result of voltage-driven conformational changes because it is observed in the absence of a transmembrane voltage. The traces in Figure 3B were obtained on the same patches as in Figure 3A but at a clamped potential of 0 mV in the presence of ionic gradient to drive the ion flow (pipette solution contains 150 mM KCl; bath solution contains 10 mM KCl).

The pattern of activity of the D315A mutant is very different from that of wild-type. The frequency of closures (upward deflections from the baseline) is notably increased. Closures are prolong and interrupted by an intense gating activity to levels of intermediate sizes. A bursting activity is not seen in wild-type channels unless they are modulated by polyamines (Iyer and Delcour, 1997). As indicated on the expanded traces in Figure 4, the top level reached by the prolong closures is not always the same. At a pipette voltage of −60 mV, most prolong closures dwell at a current level of 9.6 pA away from the baseline, while some are at a current level of 8 pA. The difference between these two levels is 1.6 pA, a value close to the current of single wild-type pore-forming subunits at this voltage (1.8 pA). Dashed lines in Figure 4 indicate current levels corresponding to a multiple of this value. It appears that many re-openings during the prolong closures have sizes of multiples of 1.6 pA. Three interpretations are possible: (1) the current through each pore-forming subunit is 1.6 pA and
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Fig. 4. Current trace of the D315A mutant channel obtained at a pipette voltage of ~60 mV and shown on an expanded time-scale. Upward deflections represent closures. Dashed lines mark current levels that are multiples of 1.6 pA away from the baseline (BL). Note that the maximum levels reached by the two main transitions are five and six times 1.6 pA away from the baseline.

multiple pores often open and closed simultaneously; (2) 9.6 pA is the current through a single pore-forming subunit that can also open and close to six different substates; (3) 9.6 pA is the current through a trimer, and each subunit displays a fully open state and a substate that is half the size of the fully open state. The available data do not allow us to distinguish between these possibilities. A cooperative behavior that extends beyond trimers is, however, not uncommon in porins (Schindler and Rosenbusch, 1981; Delcour et al., 1989b; Berrier et al., 1992; Delcour, 1997; Samartzidou and Delcour, 1998), and may be related to the high degree of porin clustering, even in the reconstituted liposomes (Delcour, 1997). Some variability exists from patch to patch, and occasionally from voltage to voltage within the same patch. Most often, prolong closures dwell at current levels that are six times the smallest size. However, the overall pattern of activity was consistently observed in 21 independent experiments performed on outer membrane fractions isolated from two membrane preparations.

Disruption of a putative salt bridge

As indicated in Figure 1B, the carboxyl group of D126 at the root of the OmpF L3 loop is within salt bridge distance from the amino groups of R100 and R168. When D118 of OmpC, the analogous residue to D126 of OmpF, is mutated to glutamine, a change in the activity pattern of porin channels is observed (Figure 5). Closures from the baseline level occur more frequently and are often clustered in bursts. Cooperative gating is still observed but does not appear as prominent as in wild-type. In addition, there is a slight increase in the frequency of openings from the baseline level (downward deflections). This type of gating behavior was seen reproducibly in 35 experiments performed with three membrane preparations. As for the D315A mutant, it appears that an increased activity of spontaneous transitions has been triggered by this mutation. To test the hypothesis that the disruption of putative salt bridges between D118 and arginines in the barrel wall is responsible for this change in behavior, we engineered two other mutants at the conserved arginines in OmpC, R92 and R174.

The R92Q mutation affected the gating behavior of OmpC channels, but surprisingly in a different way from the D118Q mutation. It drastically increased the gating frequency to such an extent that a favored baseline was no longer observed. This type of gating behavior was seen reproducibly in 12 experiments performed with three membrane preparations. A close inspection of the trace shown in Figure 5 reveals that the current oscillates rapidly between four main levels that may correspond to the non-cooperative closures and re-openings of three pores within a trimer (see expanded trace). Thus it appears that the disruption of the salt bridge between R92 and D118 induces a higher rate of transitions between closed and open states, possibly because of the increased flexibility of the L3 loop. In OmpF, this arginine residue (R100) might be involved in two salt bridges: (1) one with D126 of the L3 loop and (2) one with E71 of the L2 loop of the adjacent subunit (Karshikoff et al., 1994). If these salt bridges are conserved in OmpC (as R92–D118 and R92–E66), it is likely that the mutation of R92 to Q disrupted both of them, leading to a gating pattern distinct from that of D118Q. This can be tested in future experiments with the double mutants D118Q and E66Q. A hallmark of the R92Q mutant phenotype is the loss of cooperativity in the closing and re-opening events. This may be a consequence of the disruption of the putative intersubunit salt bridge with E66. An E66Q mutation would then be expected to reproduce the same phenotype. It is noteworthy that an E66R substitution in the related PhoE porin drastically weakens subunit interactions (Van Gelder and Tommassen, 1996).

In OmpC, the mutation of R174 to Q did not lead to any changes in gating activity. Although this residue is conserved
between OmpC and OmpF, it is possible that local structural differences exist between the two proteins, placing this residue out of salt bridge distance from D118 in OmpC. Homology modeling of OmpC suggests that this might be the case (E.Cantu, personal communication).

Voltage dependence of the D315A and D118Q mutants

Previous reports have documented that releasing the L3 loop from its interactions with the barrel wall does not interfere with the voltage dependence of channel closure or inactivation (Eppens et al., 1997; Phale et al., 1997). Here, we confirm this observation since both mutations failed to promote channel inactivation at voltages lower than required for wild-type channels (Delcour et al., 1989b; Berrier et al., 1992). Wild-type channels display a mild but asymmetric voltage dependence (Samartzidou and Delcour, 1998). In the experiments reported here on wild-type channels, the total number of the spontaneous closures was increased on average by 18 and 61% when the pipette voltage was switched from −30 to −80 mV and from −60 to −120 mV, respectively (n = 3). The voltage sensitivity of the D118Q mutant remained essentially the same, with an average 21% increase in the total number of closures upon pipette voltage change from −30 to −80 mV (n = 3). In agreement with previous findings (Phale et al., 1997), we found that the D315A mutants are less voltage dependent than wild-type, with only an average 10% increase in closures upon a pipette voltage change from −60 to −120 mV (n = 2). We also found some variability amongst the patches on D315A porins, with some of them showing a decreased gating activity at negative pipette potentials. We do not have an explanation for these findings. Possibly they originate from some variability in the orientation of the reconstituted membranes for this particular mutant.

Discussion

Porins are one of the two ion channel types whose three-dimensional structure has been determined at atomic level by X-ray crystallography (Jap and Walian, 1996). This structural knowledge can be exploited in the design of site-directed mutations and in the analysis of mutant phenotypes. Because of this tremendous advantage, a number of studies aimed at defining relationships between structure and function in ion channels have recently flourished on porins (Bishop et al., 1996; Saint et al., 1996; Eppens et al., 1997; Phale et al., 1997; Van Gelder et al., 1997a,b; Bainbridge et al., 1998). These studies have clearly established the importance of the so-called ‘eyelet’ or ‘constriction zone’ formed by the folded L3 loop across the channel opening in three major aspects of channel behavior: conductance, selectivity and voltage dependence. Although this region houses the sensor residues that confer voltage dependence on the porin channels, L3 loop motion does not appear to be responsible for the channel closure that results from applied high voltages (Eppens et al., 1997; Phale et al., 1997; Bainbridge et al., 1998).

The work described here represents a novel aspect in the search for correlations between structure and function in porins. We have focused on the rapid fluctuations that channels spontaneously make between open and closed states and that can be readily detected with the electrophysiological patch-clamp technique. The events are characteristic of all channel types and can be seen in planar lipid bilayers, but have not been the subject of previous studies. This type of activity does not require a transmembrane potential and is not the result of protein breakdown. In our studies, proteins are not purified and the conditions for reconstitution are mild and simply based on the spontaneous fusion of native vesicles with artificial liposomes. In addition, this type of activity is identical with that observed when giant cells are subjected to patch-clamp in vivo (Buechner et al., 1990). It is completely reproducible across experiments and does not represent artifactual interactions between channels and their environment. We believe that it represents the rapid conformational changes of channels between functionally distinct states.

The electrostatic calculations of Karshikoff et al. (1994) suggest that the position of the L3 loop in OmpF may be determined by three types of interactions between the loop and the barrel wall: (i) a hydrogen bond network between the tip of L3 and the adjacent barrel wall (see Figure 1), (ii) salt bridges at the root of L3 (see Figure 1) and (iii) a transverse electrostatic field between negatively charged residues on L3 and a cluster of positive charges on the opposite barrel wall. In this work, we focused on the first two types of interactions. The D315A, D118Q and R92Q mutations presented here led to an increase in the gating frequency but did not affect the voltage dependence. Our interpretation of these results is that the mutations have released the L3 loop from the interactions with the barrel via the hydrogen bond network or the salt bridges. The resulting enhanced motion or flexibility of the loop allowed for a higher rate of spontaneous transitions between open and closed states. It is also possible that the barrel has been made more flexible and is playing some role in this increased activity. Coupling between loop motion and barrel flexibility has indeed been suggested in molecular dynamic simulations of OmpF (Watanabe et al., 1997). As a corollary to the above interpretation of mutant phenotypes, we postulate that the motion of the L3 loop and/or of the barrel does exist in wild-type channels but is restricted, as reflected by the slow level of spontaneous transitions. This restricted motion is in agreement with the picture of the porin pore offered by the crystallographic studies. However, one must keep in mind that the structure revealed by X-ray crystallography is the predominant stable form under the conditions required for obtaining crystals, most likely representing one amongst various conformations. It does not exclude the existence of dynamic changes that lead to other, possibly short-lived, conformations.

It is interesting that each mutation affected the gating pattern in a distinct way. This observation argues in favor of a gating mechanism intrinsic to the channels themselves rather than a non-specific interaction between the pores and some impurities. Although we cannot offer a mechanistic explanation, we view the differences between mutants as an indication that the protein can occupy a multiplicity of conformations depending on some very local structural organizations. This suggests that the types of functional states that can be visited by the protein and the rates of fluctuations between these states may depend on small and local structural rearrangements, such as those brought about by physical or chemical modulators. Indeed, voltage (Delcour et al., 1989b; Samartzidou and Delcour, 1998), pressure (Le Dain et al., 1996), pH (N.Liu and A.H.Delcour, in preparation) and polyamines ( dela Vega and Delcour, 1995; Iyer and Delcour, 1997) greatly affect the frequency of these spontaneous transitions. Whether the spontaneous gating activity and the change introduced by these mutations affect the overall outer membrane permeability remains to be determined by in vivo assays such as antibiotic permeation.
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References


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