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Filtering with Electric Field: the Case of *E. Coli* Porins

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ABSTRACT Although the role of general bacterial porins is well established as main pathway for polar antibiotics, the molecular details of their mode-of-action are still under debate. Using molecular dynamics simulations and water as a probe, we demonstrated the strong ordering of water molecules, differently tuned along the axis of diffusion in the transversal direction. Preserved features and important differences were characterized for different channels, allowing to put forward a general model for molecular filtering. The intrinsic electric field, responsible for

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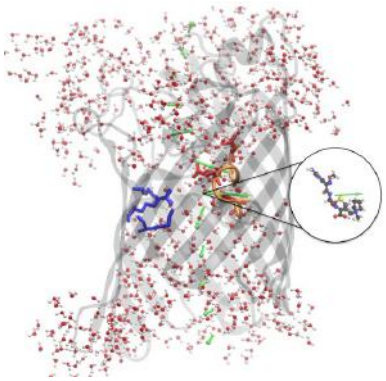
water ordering, (i) filters those dipolar molecules that can compensate the entropy decrease by dipole alignment in the restricted region, and (ii) creates a barrier by changing direction when escaping from the restricted region We tested this model using two antibiotics, cefepime and cefotaxime, through metadynamics free energy calculations. A rational drug design should take this into account for screening molecules with improved permeation properties.

TOC GRAPHICS



KEYWORDS Water, protein channels, passive diffusion, antibiotics, bacterial resistance, molecular dynamics simulations, rational drug design.

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Since X-ray structures of protein channels were available, much attention has been devoted to small specific channels, such as ion and aquaporin channels,[1-3] and to generic channels large enough to allow the passage of DNA.[4] The interest in those unspecific channels allowing the passive transport of medium-size molecules has recently arisen, for instance, toward eukaryotic VDAC, which is expressed in the mitochondrial outer membrane,[5] or the prokaryotic OmpF/OmpC family, expressed in the outer membrane of all *Enterobacteriaceae*.[6] However, function and mechanism of these β-barrel channels are still under debate.[7-9] Their classification as water-filled channels makes characterization of the interaction with water necessary. For this reason, water has been conveniently used here as a molecular probe to sense the internal electric field of OmpF/OmpC channels. These trimeric proteins serve as a general pathway for the passive exchange of hydrophilic molecules up to 600 Da across the bacterial outer membrane.[10] The internally folded loop L3 determines the overall hourglass-shape structure of each monomer, with a central constriction region (CR) characterized by a rather small size (3Å radius compared to 17Å at the mouths).[11] The observation that growth conditions characterized by high level of nutrients, such as in the mammal intestine, favor the expression of the smaller OmpC over OmpF,[10] led to the conclusion that pore size was probably the most important feature in modulating channel permeability.[11] However, numerous computational and experimental evidences are being gathered, pointing to the internal electrostatics.[12,13] The CR, indeed, is characterized by the negatively charged loop L3 that faces a positive 'basic ladder'. Recent experiments have shown that modulation of electrostatics by the medium osmolarity can account for the apparent difference in the permeability of OmpF and OmpC,[13] thus bolstering the opinion that electrostatics might play a key role in filtering. Understanding the key features responsible for permeability and selectivity of these Omp channels is fundamental, as they represent the

main access to the bacterial cell for hydrophilic antibiotics from different classes, like β-lactams.[10] One of the pathogen strategies for drug resistance is to limit the uptake by modifying the porins and/or their relative expression.[14] It is now widely recognized that our inability to come up with novel effective antibiotics for Gram-negative pathogens mostly relies upon the insufficient comprehension of the molecular basis behind penetration through the outer membrane.[15] Studies have mostly focused on the CR of porins but a consistent picture is still missing.[8,9,16-18] Finding the translocation 'golden rules' appears chimeric. Despite the unspecific character of this channel family, the available results suggest that permeation rates depend on quite general antibiotic physicochemical properties such as size, charge, hydrophobicity and flexibility, without any evident correlation.

Some theoretical investigations are already reported in the literature, aimed at characterizing the electrostatics of water inside channels[19] and of OmpF in detail. On the basis of a macroscopic multi-dielectric model, Karshikoff et.al[20] calculated a transverse electric field up to a maximum value of 36 mV $Å^{-1}$ at the CR. A full atomistic description of ion flow through the OmpF trimer with molecular dynamics (MD) simulations revealed a distinct pathway for anions and cations.[21] Full-atom, though rather short, MD simulations[22] of the transport of small polar molecules have emphasized the existence of a strong transverse electric field halfway through the porin. In the present work, MD simulations in the microsecond time-range of the whole trimer embedded in a lipid membrane are presented, aimed at analyzing the internal electrostatics of different porins in detail, namely, OmpF, OmpC and the two mutants OmpC20 and OmpC33, the first and the last one of a clinical series,[6] respectively. Interestingly, the structures of the OmpC mutants showed essentially an unchanged size,[6] offering a rare opportunity to investigate possible modulations of the internal electrostatics in correlation with the measured altered antibiotics permeability.

Because water completely fills these channels and possesses a large dipole moment though its small size, it represents the natural probe for channels' internal electrostatics and the electric field experienced by diffusing molecules. Without restricting the attention to the CR but looking at the entire lumen, we could reveal a remarkable water 'choreography' across the channel, with striking differences among the four porins investigated, particularly at the interface between the extracellular vestibule (EV) and the CR.

The multiple-structure alignment of the four porins under investigation (Figure S1) shows some interesting features: (i) insertions resulted only in extracellular loops of different length, (ii) the majority of charged residues mutations are observed in the extracellular loops, and (iii) at the CR, both the loop L3 and the 'basic ladder' are highly conserved. This clearly suggests that CR electrostatics is comparable in OmpF and OmpC and it is not significantly altered throughout the series of OmpC mutants, isolated from increasingly resistant *E. coli* clinical strains.[6] This feature appears the fundamental structural determinant for correct filtering of nutrients and hard to be significantly mutated. Specific mutations are consequently due in other regions of the channel, allowing for filtering out noxious species but allowing the passage of desired nutrients at the same time. In order to analyze the net charge distribution, channels were positioned (their center of mass) in the center of the reference system with the diffusion axis oriented along z, and then split into three sections, namely, the EV ($z > +10$Å), the CR ($+10$Å $\leq z \leq -10$Å) and the periplasmic vestibule (PV; $z < -10$Å). All the investigated porins have a net negative charge (Figure S2), which increases from OmpF to OmpC and is even larger in the two mutants OmpC20 and OmpC33. As already mentioned, CR was found to be conserved. This is the more negative section of all the four porins and its net charge appears hard to be altered. Only a -1 difference was found in OmpC33, the last one of the clinical mutants series.[6] Quite surprisingly,

the largest differences pertain to the EV, which is more negative in OmpC than in OmpF, and whose net negative charge is further increased in the clinical mutants. The same trend was found for the PV, despite the differences being less pronounced. It is also interesting to note that the PV is more negatively charged than the EV in the two wild-type porins, while, in the two clinical mutants, residues mutations led to the same net charge for both the vestibules.

In order to characterize and quantify such an altered electrostatics inside the lumen and to examine the effect of the porin's intrinsic electric field, the electric dipole $\boldsymbol{\mu}$ of the water molecules has been used as order parameter along the MD trajectory. To this aim, the protein channel was divided into 12 adjacent sections of identical thickness along the main axis of the pore (z-windows). For each z-window the total $\boldsymbol{\mu}_t$, due to all the water molecules N_w present in that z-window, was computed. Then, the normalized electric dipole $\boldsymbol{\mu}_t/\mathbf{N}_w$ in each z-window was obtained for each of the 6,000 MD simulation frames (corresponding to 300 ns) and its time average was calculated. Bulk waters are characterized by a rather short correlation-time for reorientation, such that the corresponding autocorrelation function decays to zero rapidly. Conversely, ordering of water molecules by the internal electric field of the porin resulted to be a non-Markovian process. The function $\mu_t/N_w(t)$ did not decay to zero but asymptotically tended to a constant value which depended upon the specific z-window considered.

Figure 1 shows a cartoon representation of the first monomer of each of the four porins, together with the vectors corresponding to the time averaged $\boldsymbol{\mu}_t/\mathbf{N}_w$. It is immediately evident how, in all the cases, the maximum water ordering is reached halfway through the channel (z-window 7, from the top of Figure 1), with the dipole pointing the loop L3 from the 'basic ladder'. This dipole corresponds to a transverse electric field as it has been already shown for OmpF.[20,21]

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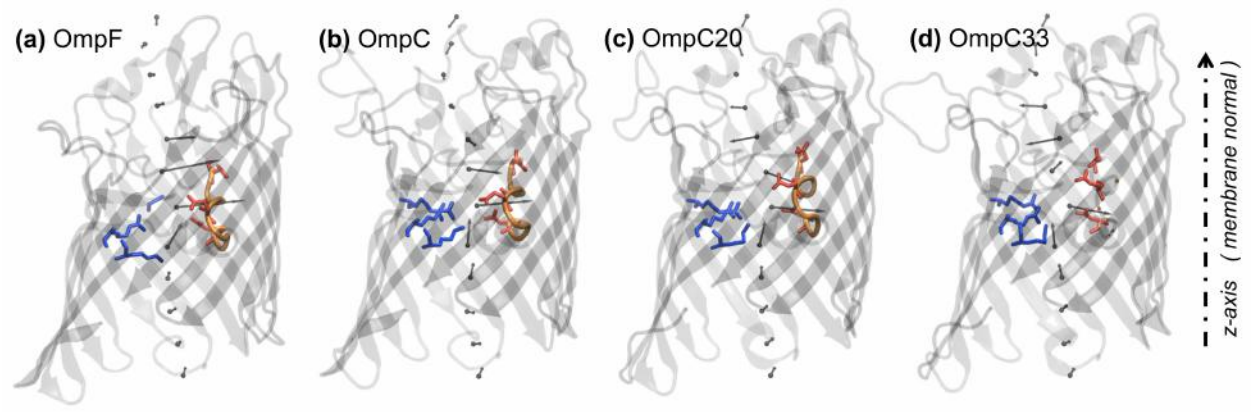

Figure 1. Dipole water orientation in **(a)** OmpF, **(b)** OmpC, **(c)** OmpC20 and **(d)** OmpC33 from *E. coli*. A cartoon of the first monomer is shown together with the positively and negatively charged residues of the 'basic ladder' and loop L3 (orange), respectively. The former are reported in blue, the latter in red. Vectors represent the time average of the total dipole moment $\boldsymbol{\mu}_t$ of the water molecules found in the corresponding protein section, normalized by the number of water molecules N_w.

To better appreciate and quantify differences and similarities among the four porins, in Figures 2a-2d we plotted the dipole strength μ_t/N_w in each z-window, and in Figures 2e-2h its projection on a plane perpendicular to the diffusion axis for the z-windows close to and at the CR, i.e. from z-window 5 to 8. In the z-window 6 the water dipole has almost the same direction as in the z-window 7, but moving from OmpF to OmpC and, then, to the OmpC mutants, intensity decreases and a progressive difference in the xy component is observed.

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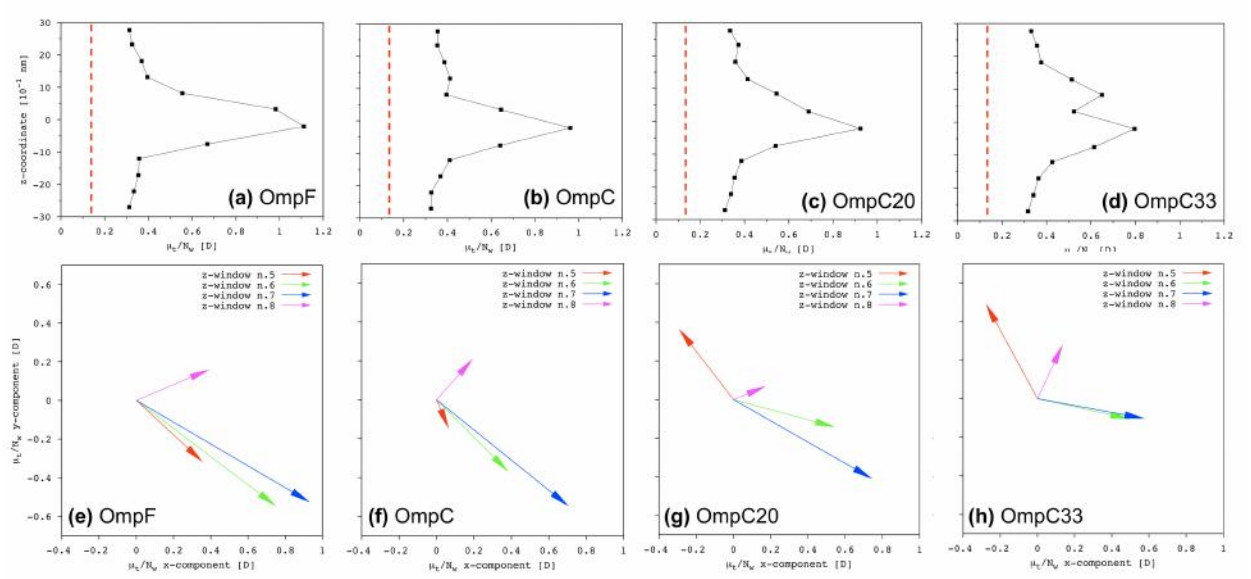

Figure 2. (a-d) The μ_t/N_w module profile is shown along the channel axis. The red dashed line represents the value obtained for bulk water (0.12 D). In **(e-h)** the xy-projections of the μ_t/N_w vectors pertaining to the central z-windows of the protein channel are reported.

While all the porins are characterized by a conserved significant variation of the waters net dipole upon emerging from the CR to the PV (~ 90°; z-window 7 to 8), the differences found at the level of the z-window 5 are absolutely remarkable. In OmpF the μ_t/N_w vector has almost the same direction as in the z-windows 6 and 7. The same direction is preserved also in OmpC but water dipole strength is dramatically reduced. Astonishingly, water dipole direction is almost inverted in OmpC20 and the same was found in OmpC33 with an even higher strength. These profiles clearly show that the transverse electric field, as evaluated using the dipole moment of water molecules, is maximum at the CR in all the investigated porins. The observation that this particular feature was not modified in the drug resistant clinical strains[6] points once more to the importance of the electrostatics for the correct porin function as a filter for nutrients uptake.[13] However, while only a slight difference was observed in the maximum value of μ_t/N_w (Figure 2a-

2d) in OmpF (1.11 D), OmpC (0.96 D) and OmpC20 (0.92 D), it is interesting to note that in the last clinical mutant, OmpC33, the waters net dipole significantly decreased (0.80 D) and became much more disordered in the CR (Figure 1d), as it was already put forward to explain the apparent decreased susceptibility to antibiotics.[6] However, the most striking difference among the investigated porins pertains to the transit from the EV to the CR. The ordering of water dipoles in each channel is determined by the specific distribution of the positively and negatively charged residues inside the lumen,[5,13,21] as clearly shown by the detailed analysis reported in the SI. The overall emerging picture is that specific mutations have been introduced to tune the transversal electric field right at the entrance of the CR (z-window 5), while leaving the CR almost unaffected. Upon entering the CR, the translocating molecule experiences a remarkable decrease of the conformational entropy, due to the significant pore size reduction. The resulting increase of the free energy would be compensated by the favorable orientation of the molecular electric dipole with respect to the channel transverse electric field. Thus, the effect of a weak field just above the CR (e.g. in OmpC) or, even worst, of an electric field pointing in the opposite direction (e.g. in the two OmpC mutants) is expected to impose an adverse 'pre-orientation' to the translocating molecule, exactly where steric hindrance to molecular reorientations starts to be severe. The same can be applied to the conserved waters order deviation observed upon emerging from the CR to the PV. From a general point of view, it is absolutely plausible that the internal electric field of the channel forces a dipolar molecule to align its moment accordingly while translocating. The highest free energy barriers for translocation are thus expected where large molecular reorientations are needed in constricted regions of the pore.

Because of the usually high negative net charge of the CR of bacterial general porins, like those investigated in the present work (Figure S2), permeation is widely believed to be easier for

zwitterionic than for negatively charged antibiotics. This is absolutely reasonable, since the presence of a net positive charge on the translocating molecule should help approaching and then entering the CR. Both experimental and computational evidences have been provided in the literature[8,9] but, for the sake of completeness, it has to be mentioned that other authors have found only low correlation between electrophysiology measurements and antibiotics net charge.[16,23] The present investigation has shown a rather complex water 'choreography' across the channel and suggests the electric dipole moment as a more general molecular feature to be looked at when comparing different antibiotics. Quite naively, zwitterionic molecules might be preferred for a rational drug design, since a clear segregation between a positive and a negative charge would ensure a well defined electric dipole.[18] However, regardless the specific molecule is actually zwitterionic, but providing it has a comparable electric dipole, our simulations predict a general translocation mechanism. In order to preliminarily check this hypothesis, two cephalosporins were selected, namely, cefepime and cefotaxime, and the free energy surface (FES) for translocation through OmpF was reconstructed using metadynamics (details in the SI). These two molecules were chosen due to their similar size (they differ only by one side chain) but the extremely different charge distribution, the former being zwitterionic at neutral pH, the latter being negatively charged with no positively charged groups. They are both characterized by a remarkable and similar electric dipole, 20.5±1.3 D and 25.8±2.2 D for cefepime and cefotaxime, respectively (calculated in their center of mass and averaged over the conformations sampled in the MD trajectories). The most interesting difference is certainly the dipole orientation with respect to the longest axis of the molecule (Figure 3a and 3d).

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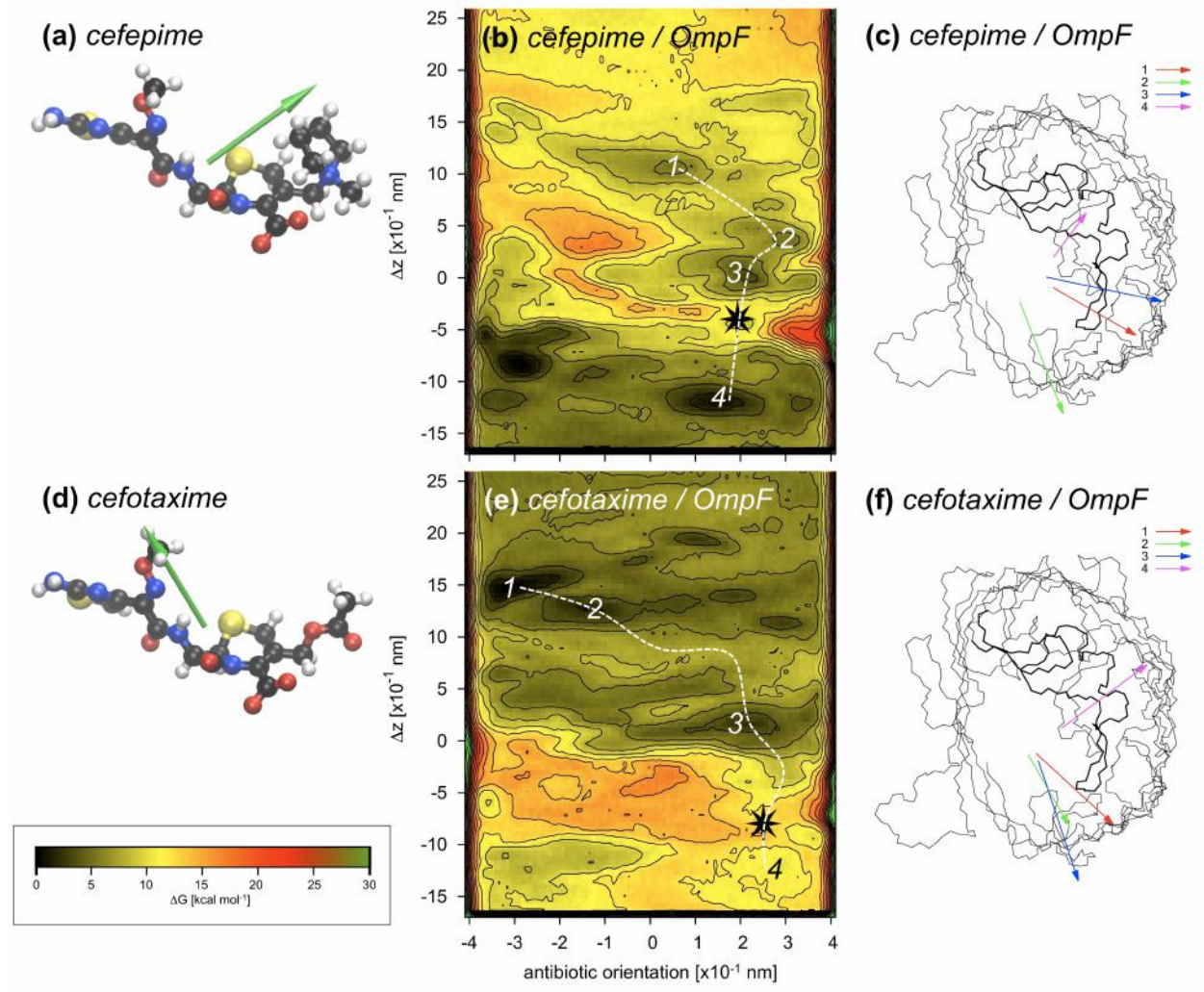

Figure 3. Cefepime **(a)** and cefotaxime **(d)** are shown together with the corresponding electric dipole (green arrow). Chemical elements are color coded as follows: C black, H white, N blue, O red, S yellow. The FES for translocation through OmpF are shown in **(b, e)**, where the main minima are labeled with consecutive numbers. The highest barrier is marked by an asterisk. Antibiotic orientation is defined as the difference of the z-coordinate between the lactam carbonyl C and the S bonded C. For each of the labeled minima, the molecular electric dipole was calculated and the average vector is reported in **(c, f)** together with the channel backbone trace. The loop L3 is bolded.

Figure 3b and 3e show the FES for the two antibiotics inside OmpF. Regardless their differences, both cefepime and cefotaxime experienced the highest energy barrier in the central region of the channel. More precisely, while approaching the CR from the EV both antibiotics are accompanied by a rather low energetic cost, up to the z-windows 6 and 7. The highest energy barrier was found between the z-windows 7 and 8, i.e. where the water analysis has revealed the unique abrupt change in the direction of the waters net dipole moment (figure 2e), upon emerging from the CR to the PV. Figure 3c and 3f show the average antibiotic dipole corresponding to the various free energy states labeled on the FES. Before entering the CR, both antibiotics are pre-oriented by the electric field at the level of the EV such that, in the case of OmpF, the molecular electric dipole has an orientation favorable to get into the CR. However, upon emerging to the PV, the direction of the channel field is significantly different and the antibiotic reorients to properly align its electric dipole. Such reorientation unfavorably occurs in the constricted region of the lumen, leading to a significant increase of the translocation free energy barrier.

In both human aquaporin-1 AQP1 and the *E. coli* aquaglyceroporin GlpF,[2,3] the intrinsic electric field of the transmembrane pore was found to be absolutely essential for both channel specificity and functionality. Water molecules and not protons permeate the channel by following a specifically designed "choreography", referred to as 'global orientational tuning',[2,3] by aligning their dipole moment to the porin's electric field that changes orientation from the extracellular to intracellular side. In the completely different context of bacterial outer-membrane general porins, which are larger than aquaporins and not selective channels, the effect of the channel electric field is shown in this work, as highlighted by the waters 'choreography' in Figure 1. When compared to the case of AQP1 and GlpF, where the direction of the water dipole

mostly changes along the channel axis,[2,3] in the present case of general porins the transverse reorientation appears to be predominant and represents the most striking difference among the examined channels, especially in the two porins extracted from clinical strains, for which a lower permeability was measured. The presented results have important implications for the formulation of a general model for antibiotics translocation. On the one hand, a common bottleneck was identified for all the investigated porins, the molecular reorientation due to a variation of the electric field upon escaping from the CR to the PV. On the other hand, the CR mouth on the extracellular side has emerged as the most critical area, susceptible to mutations and differentiating the inspected *E. coli* channels. Molecules can be unfavorably pre-oriented before entering the small CR. Then, when forced to reorient in a rather small region in order to compensate the entropy decrease, steric hindrance to such reorientations might became the main contribute to translocation free-energy barrier. Moving from the concept that zwitterionic molecules should be favored[18] to the more informative suggestion that the molecule's electric dipole moment strength and orientation do determine permeability, a rational drug design should take this into account. Looking at the electric dipole moment of molecules rather than to their mere zwitterionic character, the ability to reorient and modulate the intensity of the electric dipole without the need of sterically hindered rotations is suggested as a promising route.

ASSOCIATED CONTENT

Supporting Information

Computational methods. Figure S1: multiple structure alignment. Figure S2: Net charge distribution. Figure S3-S5: Charged residues distribution for different protein sections is compared with the total dipole moment of the water molecules. Figure S6: The important amino

acid mutations responsible for the different orientation of the water dipole moment at the extracellular mouth of the constriction region. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interests.

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