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Antibiotic Permeation across the OmpF Channel: Modulation of the Affinity Site in the Presence of Magnesium

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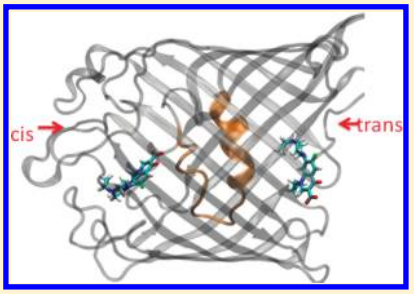
Supporting Information

ABSTRACT: We characterize the rate-limiting interaction of the antibiotic enrofloxacin with OmpF, a channel from the outer cell wall of *Escherichia coli*. Reconstitution of a single OmpF trimer into planar lipid membranes allows measurement of the ion current through the channel. Penetration of antibiotics causes ion current blockages, and their frequency allows a conclusion on the kinetics of channel entry and exit. In contrast to other antibiotics, enrofloxacin is able to block the OmpF channel for several milliseconds, reflecting high affinities comparable to substrate-specific channels such as the maltodextrin-specific maltoporin. Surprisingly, the presence of a divalent ion such as Mg^{2+} leads to fast flickering with an increase in the rates of association and dissociation. All-atom computer modeling provides the most probable pathway able to identify the relevant rate-limiting interaction during antibiotic permeation. Mg^{2+} has a high affinity for the aspartic acid at the 113 position (D113) in the center of the OmpF intracellular binding site. Therefore, the presence of Mg^{2+} reverses the charge and enrofloxacin may cross the constriction region in its favorable orientation with the carboxylic group first.



INTRODUCTION

Gram-negative bacteria account for half of the life-threatening infections in clinics today, and in particular, increasing antibiotic resistance is an emerging problem. The first line of defense against antibiotics is the outer cell wall. Water-soluble antibiotics cross this barrier through membrane channels called porins. For example, β-lactams and quinolones have been commonly used for the treatment of bacterial infections.[1−5] β-Lactams act on the peptidoglycan layer that is located between the outer and inner bacterial membranes, whereas quinolones target the DNA−topoisomerase complex in bacteria.[1−5] To cross the outer membrane, the β-lactams and quinolones must pass through porins located in the outer membrane. In *Escherichia coli* two porins—OmpF and OmpC—are considered as a major pathway for antibiotic translocation.[4,5] Both OmpF and OmpC channels are homotrimers, and in each monomer 16 β-strands span the outer membrane to form a barrel.[6,7] Downregulation of these porins as well as point mutations can lead to reduced accumulation of quinolones (and other agents) within the cell.[2−4] OmpF facilitates the diffusion of fluorinated quinolones such as norfloxacin and the cephalosporins such as cefoxitin into the periplasm, with marked reductions in accumulation of these agents in the OmpF-deficient bacterial strains.[8,9] Therefore, quantifying the barrier for influx through porins might contribute to the complex question of multiple-drug resistance.

Recently we applied ion current fluctuation analysis on a single-channel level to gain insight into the interaction of solutes with the channel interior.[10−12] Permeation of molecules inside the channel causes fluctuations in the ion current, reflecting the particular interactions with the channel wall. The analysis of the ion current fluctuation allows permeation rates to be obtained as previously shown for sugars and antibiotics.[10−12] As previously discussed, this approach has a number of limitations.[13] First, ion current fluctuations can only be used as a signal for binding under the condition that the ion current occlusion during binding is strong enough to be recorded. However, binding does not imply translocation. Moreover, the time resolution is limited, and events faster than 0.1 ms will not be detected.[13] In the case of charged molecules, the external field is an additional driving force; increasing the force should lead to faster translocation. In particular, for peptides, the residence time as a function of applied voltage can be used as a probe for true translocation.[14] However, in the case of zwitterionic substances, a further method to evaluate translocation is needed.[13] For example, molecular modeling provides details on the interaction of the molecules with the channel surface and reveals the preferred orientation of the antibiotic along its pathway.[11,16]

Here, we investigate the interaction of the fluoroquinolone enrofloxacin with OmpF. In particular, we study the effect of applied transmembrane voltage and the presence of divalent ions,

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in particular magnesium chloride, on antibiotic interaction. The hydrophobicity of enrofloxacin is very high, and its structure is shown in the inset in Figure 1. To allow a molecular

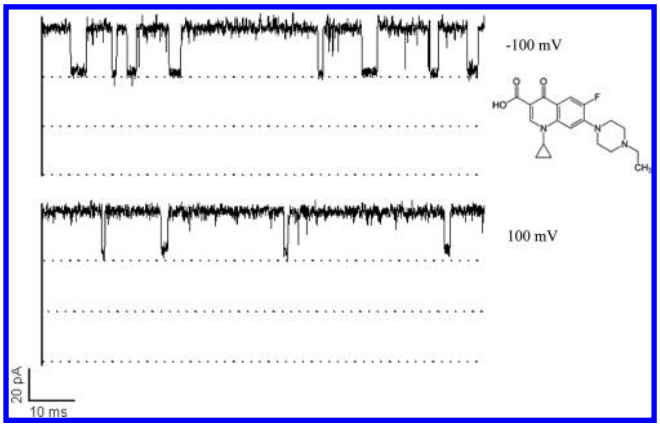

Figure 1. Ion current through a single OmpF trimeric channel in the presence of 1 mM enrofloxacin (cis side) at −100 and +100 mV. Ion current blockage events strongly depend on the polarity of the applied voltage. Reagents and conditions: 150 mM KCl, 5 mM MES, pH 6. The neutral structure of enrofloxacin is shown in the inset.

interpretation of the conductance measurements, we complement our study with all-atom molecular dynamics simulation. This allows identification of the antibiotic affinity sites in the channel and associated rate-limiting interactions.

EXPERIMENTAL SECTION

The following chemical reagents were used in this study: NaCl, KCl, $MgCl_2$, MES, *n*-pentane, and hexadecane (Sigma), enrofloxacin (Fluka), *n*-octylpoly(oxyethylene) (octyl-POE) (Bachem, Bubendorf, Switzerland), and 1,2-diphytanoyl-*sn*-glycero-3-phosphatidylcholine (DPhPC) (Avanti Polar Lipids, Alabaster, AL). Doubly distilled and deionized water was used to prepare all solutions.

Single-Channel Conductance Measurements. Virtually solvent-free planar lipid membranes were formed using diphytanoylphosphatidylcholine (DPhPC) (Avanti Polar Lipids, Alabaster, AL) according to the Montal−Mueller technique.[15] The measurements were carried out with standard buffer containing 150 mM KCl and 5 mM MES, pH 6.0, that serves as an electrolyte. Complementary experiments contained in addition 5 mM $MgCl_2$. A Teflon cell with an approximately 50−100 μm diameter aperture in the 25 μm thick Teflon partition was used together with standard silver−silver chloride electrodes from WPI (World Precision Instruments). Small amounts of OmpF porin from a diluted stock solution of 1 mg/mL were added to the cis side of the chamber (side connected to the ground electrode). Spontaneous channel insertion was usually obtained with stirring under an applied voltage. Conductance measurements were performed using an Axopatch 200B amplifier (Axon Instruments) in the voltage clamp mode. Signals were filtered by an on-board low-pass Bessel filter at 10 kHz and recorded onto a computer hard drive with a sampling frequency of 50 kHz. Amplitude, probability, and noise analyses were performed using Origin (Microcal Software Inc.) and Clampfit (Axon Instruments) software. The enrofloxacin stock solution for conductance measurements was made at a final concentration of 2.0 mM in 150 mM KCl, 5 mM $MgCl_2$, and 5 mM MES (pH 6.0). Enrofloxacin was added to the cis and trans sides of the chamber to investigate their permeation rates through the porin channels. Kinetic parameters of the enrofloxacin binding for the asymmetric and symmetric drug addition were calculated by single-channel analysis. The association rate constant k_{on} gives the permeation of the antibiotic molecule from the cis or trans side to the affinity site in the channel. The dissociation rate constant k_{off} gives the rate at which antibiotic molecules were released from the channel affinity site to the cis or trans aqueous phase. The equilibrium binding constant K (ratio of k_{on} to k_{off}) defines the affinity of the drug for the porin molecule.[10−12]

Molecular Dynamics Simulations. The starting structure for the OmpF channel was prepared as shown previously.[16] We used one monomer of the crystal structure (PDB code 2OMF) that was resolved at a resolution of 2.4 Å. We embedded the system in a hydrophobic environment of detergent molecules (lauryldimethylamine oxide, LDAO) and solvated the system with ∼8000 water molecules; we used the Amber potential and TIP3P for water. The force field for the enrofloxacin antibiotic was derived following the Amber force field rules with charges obtained from the Gaussian program (6-31G** basis set) on the optimized geometry of the molecule. We performed MD simulations with the Orac[27] program: we simulated a periodic box in the NVT ensemble using the Nose thermostat (300 K), a 10 Å cutoff, and soft particle mesh Ewald (SPME) (fifth order, grid less than 1 Å in size) for electrostatic interactions and the multiple-time-step algorithm (time steps of 0.5, 1.0, 2.0, 4.0, and 12 fs).

Using the metadynamics algorithm, we employed MD simulations to reveal the translocation mechanism of enrofloxacin through the OmpF channel.[16] The metadynamics algorithm allows the reconstruction of the free energy in the subspace of the collective variables by integrating the history-dependent terms. Molecular dynamics simulations of the enrofloxacin−OmpF complex started with the antibiotic added on the extracellular side at 10 Å from the constriction region, as discussed previously.[16] We added a magnesium ion near the constriction region, substituting for a potassium ion there, and to maintain the box neutral, we eliminated an additional potassium ion. For the magnesium ion, we used the standard Amber parameters[17] derived from free energy perturbation simulations (R^* = 0.7926 Å, ε = 0.8947 kcal/mol). Recently new parameters were obtained by fitting the osmotic pressure,[26] and the main problem was representating ions as a uniformly charged sphere. We allowed the system to equilibrate from 100 to 300 K for 1.5 ns before starting the metadynamics simulations. As shown previously,[16] we used two collective variables: (1) the angle θ, the orientation of the enrofloxacin long axis (almost parallel to the dipole moment) with respect to the z axis of OmpF, and (2) the distance Z, the projection along the z axis of the position of the center of mass of enrofloxacin with respect to the center of mass of OmpF. We added with a frequency of 4 ps a bias to force the system to sample more efficiently the subspace of the two collective variables, a repulsive Gaussian potential of 1.0 kJ/mol height and 5.0° and 0.4 Å width, respectively, for the two variables. The choice of these parameters allows an equilibration of the other degree of freedom and the reconstruction of the free energy with an error not exceeding 2 kcal/mol.[16] We used a single metadynamics trajectory allowing enrofloxacin to go from the extracellular space to the periplasmic space to identify the free energy minima in the space of the two collective variables, the angle θ and distance Z. Additional standard MD

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simulations were performed with enrofloxacin in the different minima to analyze its specific interactions.

RESULTS AND DISCUSSION

Ion Current Blockage To Reveal On and Off Rates. To quantify the kinetics of enrofloxacin interaction, we reconstituted a single OmpF trimer in artificial planar lipid membranes. Studying OmpF channels at different voltages reveals a slight asymmetry with respect to ion conductance. For example, if the porin is added to the cis side of the chamber, the single-channel conductance is slightly higher (G = 1.0 nS) at positive voltage compared to negative voltage (G = 0.9 nS). As we obtain a strong correlation of this asymmetry with the side of protein addition, we use this feature as a test for the direction of the channel insertion.[12,16] Addition of enrofloxacin to the system caused transient blockage of the ionic current through a single trimeric OmpF channel in a voltage-dependent manner. Figure 1 shows that penetrating enrofloxacin interacts with the OmpF channel, resulting in channel blockages. At low drug concentration, enrofloxacin interacts with the OmpF channel, resulting in monomer blocking, and at increasing concentration, dimer and trimer blocking is visible.[16]

The number of blockage events and average residence time were calculated by ion current fluctuation analysis. Figure 2

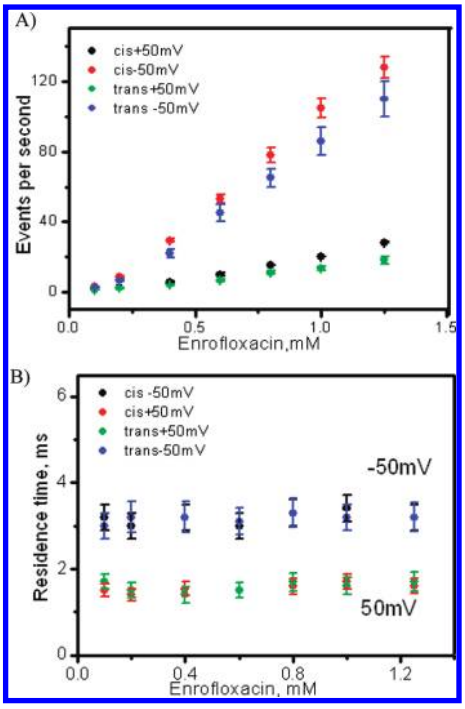

Figure 2. (a) The number of events is linear with the concentration of the enrofloxacin used and depends on the applied voltage. (B) The average residence time does not depend on the concentration of drug but the applied voltage.

summarizes the number of events and residence times of penetrating enrofloxacin under various concentrations and applied voltages. Surprisingly, Figure 2 shows high asymmetry in the residence time and blocking events with respect to the polarity of the applied transmembrane potential. At negative voltages, blockages are 10 times more frequent than at positive voltages irrespective of the concentration of the antibiotic used (Figure 2A). Single-channel analysis revealed an exceptionally long residence time for enrofloxacin in the OmpF channel. The average residence time of enrofloxacin strongly depends on the applied voltage but not on the concentration of antibiotic used (Figure 2B). The average residence time was calculated to be 3.5 ± 0.5 ms at −100 mV and 1 ± 0.3 ms at +100 mV. Such a long residence time is a result of strong interaction between the penetrating antibiotic and the binding site in the porin and is approximately 1 order of magnitude higher than those for various β-lactams.[10,11,18] Previously, it has been shown that wild-type OmpF has two symmetric binding sites for enrofloxacin located at each channel entry separated by a large energy barrier in the center and the ion current blockages are caused by enrofloxacin molecules occupying either one of these peripheral binding sites.[16] However, this longer residence time of enrofloxacin does not imply an effective translocation.[16,19] The affinity constant of enrofloxacin in the case of OmpF is comparable to the specific affinity of maltooligosaccharides to maltoporin.[12] The ability of enrofloxacin to specifically block the ion currents through the OmpF channel for a longer time can be used to identify this channel.

In a second series of measurements, we investigate the effect of magnesium ion on the interaction of enrofloxacin with OmpF. Surprisingly, addition of magnesium causes a dramatic change in the enrofloxacin binding kinetics. Figure 3 shows the

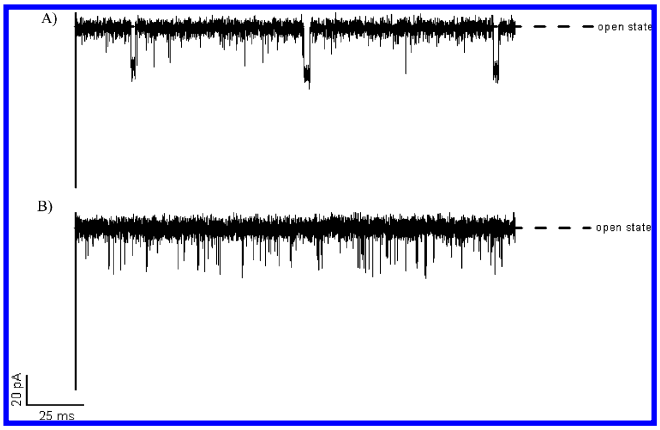

Figure 3. Enrofloxacin (100 μM) induced ionic current fluctuations through a single OmpF channel in the (A) absence and (B) presence of magnesium chloride. Reagents and conditions: 150 mM KCl, 5 mM $MgCl_2$, 5 mM MES, pH 6, 100 mV.

ion current blockage events of enrofloxacin through a single trimeric OmpF channel in the presence and absence of magnesium chloride. In the presence of magnesium, enrofloxacin (cis side addition) blocks the ion current through the OmpF channel with fast flickering events. The effect observed in the presence of $MgCl_2$ strongly depends on the polarity of the applied voltage and the side of drug addition. The number of enrofloxacin blocking events increased and the average residence time decreased at positive voltages but not at negative voltages. The effect of interaction is stronger if enrofloxacin is added to the trans side of the chamber. The strong blocking at trans side addition does not allow individual blocking events to be distinguished but causes rather a reduction in the ion conductance. To resolve individual blocking events, we lowered the concentration of enrofloxacin to 5 μM. As shown in Supplementary Figure 1 (Supporting Information), we observed clear monomer blockage events. The average residence time of enrofloxacin in the OmpF was calculated to

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be 1.2 ± 0.3 ms in the absence of magnesium ions and 100 μs in the presence of magnesium chloride at 100 mV (Figure 4).

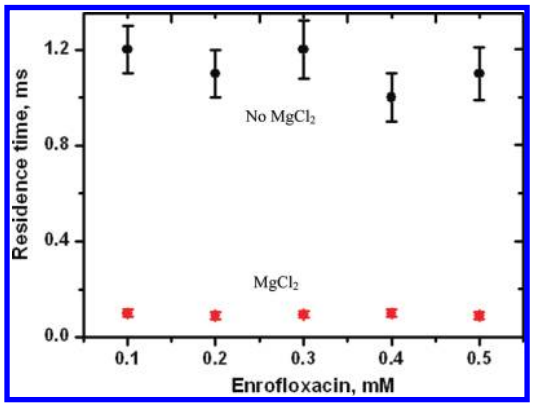

Figure 4. Average residence time of enrofloxacin through the OmpF channel in the absence and presence of magnesium chloride at different antibiotic concentrations. Reagents and conditions: 150 mM KCl, 5 mM $MgCl_2$, 5 mM MES, pH 6, 100 mV.

Kinetic constants and on and off rates of enrofloxacin binding to the OmpF channel in the absence and presence of magnesium chloride are presented in Table 1.

Free Energy Profiles and Rate-Limiting Interactions. The crystal structure of the OmpF channel in the presence of 1 M $MgCl_2$ shows that the Mg^{2+} cation was bound between residues D113 and E117.[20] Although the conditions used there are not specific to investigation of the binding of ions, previous theoretical and experimental investigations confirmed a specificity of this region to interact with divalent cations.[21,25] In our simulation, the Mg^{2+} interacts directly with two oxygens of the protein and with four water molecules. We reconstructed the free energy surface using our reactive metadynamics path with enrofloxacin that moves from the extracellular to the periplasmic space (Figure 5A). We identified two minima, the first in the extracellular side, at position (θ = 160°, Z = +7 Å), and the second near the constriction region and shifted more toward the periplasmic space, at position (θ = 130°, Z = −4 Å). The latter is not present in the simulation without magnesium ion[16] (Figure 5B), where we identified a minimum with a different orientation (θ = 50°, Z = −4 Å). From the reconstructed free energy surface, we evaluated the effective barrier to translocate from the extracellular to the periplasmic space at 10 kcal/mol, to be compared to 15 kcal/mol without magnesium (Figure 5 C,D). In Figure 6, we report a snapshot of enrofloxacin in the new central minimum. We calculated the area accessible to the solvent when enrofloxacin occupies this affinity site, and it has a value of 6.2 $Å^2$, while the minimum dimension of the pore is on average 26.3 $Å^2$, only 23% availability of the total space, which explains the current blockages observed experimentally. The enrofloxacin transverses the constriction region with the carboxylic group pointing down, and the same orientation is kept in the central affinity site with the hydrophobic group, which carries the positive charge, close to the L3 loop (Figure 6). It is important to note that in our simulation there is no external electric field biasing the orientation of enrofloxacin during translocation. Interestingly, in our simulation, the Mg^{2+} ion, positioned near the L3 loop, is able to screen completely the carboxylic group of residue D113, producing local charge inversion.[25]

We show that, in the presence of magnesium chloride, enrofloxacin has completely different binding kinetics for the OmpF channel. The antibiotic blocks the channel with more frequent binding events and a shorter residence time of 100 μs, which indicates that the rates for association (k_{on}) and dissociation (k_{off}) drastically increase in the presence of magnesium chloride. MD simulations show that enrofloxacin now can traverse the constriction region with the carboxylic group pointing down, while without magnesium this orientation is inhibited by the strong repulsion with the aspartic acid at the 113 position (D113). The position of the magnesium ion allows the complete screening of D113 and increases the probability of antibiotic reaching the central affinity site starting from the cis side. The antibiotic crosses the constriction region and further translocates through the channel. Interestingly, the enrofloxacin binding kinetics in the presence of magnesium strongly depends on the polarity of the applied voltage. The increase in k_{on} and k_{off} was observed only at positive voltages (Table 1). The positive voltages promote the orientation of enrofloxacin with the carboxylic group pointing down. This is also the position assumed by enrofloxacin at the central affinity site; see Figure 5A. However, at negative voltages, enrofloxacin orientation has the carboxylic group up and it is not favored to occupy the new central affinity site. In our study, we observed ion current blockage in channels when enrofloxacin was added asymmetrically to either the cis or trans side of the channel. When the antibiotic was added to the trans side (periplasmic side) of the chamber, the binding events were about twice as frequent as when the antibiotic was added to the cis side (extracellular side) in the presence of magnesium chloride (Table 1). This is also suggested by inspection of the free energy surface of Figure 5A: Because the central affinity site is more shifted toward the trans side, the addition of antibiotics here favors its occupation, resulting in faster kinetics. On the other hand, when the antibiotic is added on the cis side, it has to traverse the constriction region before it can occupy the central affinity site, with a barrier that we calculated to be 10 kcal/mol.

Table 1. Association (k_{on}) and Dissociation (k_{off}) Rate Constants and Equilibrium Constant (K) Obtained by Single-Channel Conductance Measurements on OmpF[a]

	k_{on}^{cis} (M^{-1} s^{-1}) × 10^3	k_{on}^{trans} (M^{-1} s^{-1}) × 10^3	k_{off}^{total} (s^{-1}) × 10^3	K (M^{-1})
+100 mV	4 ± 0.3	4 ± 0.3	0.7 ± 0.05	11 ± 1
+100 mV ($MgCl_2$)	2100 ± 200	26000 ± 2000	11 ± 1	2500 ± 200
−100 mV	12 ± 1	11 ± 1	0.29 ± 0.02	80 ± 7
−100 mV ($MgCl_2$)	11 ± 1	10 ± 1	0.30 ± 0.02	70 ± 6

[a] k_{on} = (number of events s^{-1})/(antibiotic concentration), k_{off} = 1/(average residence time), and $K = k_{on}/k_{off}$. The enrofloxacin conentration was 100 μM.

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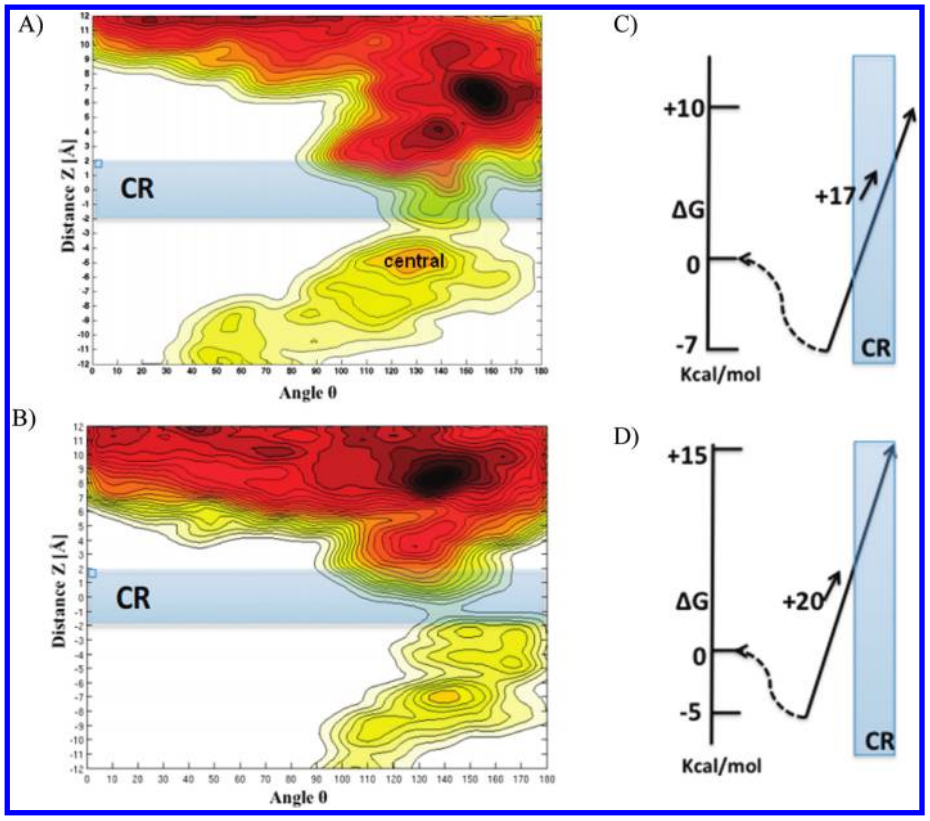

Figure 5. Reconstructed free energy surface from metadynamics for enrofloxacin traversing the OmpF channel in the presence of magnesium (A) and without magnesium (B). Panel B is reprinted from ref 16. Copyright 2010 American Chemical Society. The two collective variables are the angle θ along the x direction and the distance Z along the y direction. Each color corresponds to a 1 kcal/mol energy difference. In panels C and D we report the 1-D minimum free energy path for the two simulations as extracted from panels A and B, respectively.

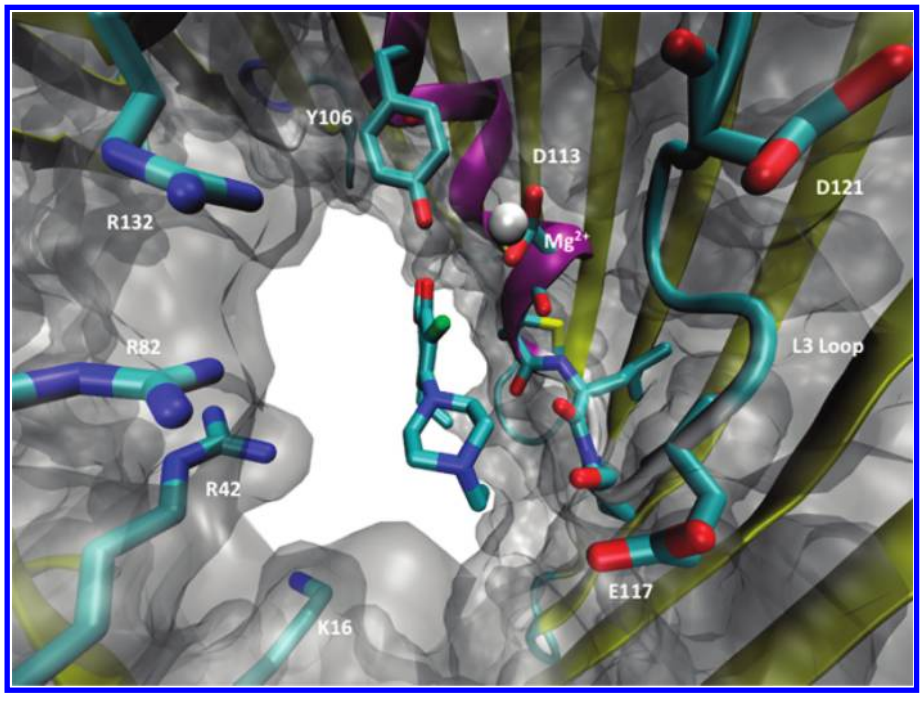

Figure 6. Molecular interaction of enrofloxacin with the OmpF channel in the preferential affinity site near the constriction region in the presence of magnesium (white sphere), showing specific interactions between enrofloxacin and the residues of the channel affinity site.

■ CONCLUSION

Ion current fluctuation analysis has often been used to characterize permeation of water-soluble antibiotics through a membrane channel. Favorable interaction increases the concentration of the drug inside the channel and enhances the number of translocation events. Combining ion current

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fluctuation analysis and atomistic computer simulations, we arrive at a picture demonstrating enrofloxacin translocation through the OmpF porin. Although most of the antibiotic shows a very low affinity for the OmpF channel, enrofloxacin is a striking exception. Pronounced blocking of OmpF channels with enrofloxacin was observed, and blocking does not imply translocation. However, the presence of magnesium chloride alters the affinity of the antibiotic for the channel wall. Binding of Mg^{2+} to residue D113 completely screens the charge, producing a local charge inversion that favors the antibiotic to cross the constriction region and further translocate with a faster kinetics. Our results reinforce the idea that OmpF works as an electrostatic filter against antibiotic translocation.[23,24] This is in agreement with a recent analysis of all marketed drugs: among these, antibiotics have the strongest polar character.[22] Translocation of antibiotics is dominated by electrostatics, and in the future trivalent ion La^{3+} could be tried to understand new physical insights into translocation.[25] Our analysis of the data provides a full quantitative description of relevant kinetic and electric parameters of enrofloxacin interaction with the channel surface in the presence of magnesium.

■ ASSOCIATED CONTENT

Supporting Information

Structure of enrofloxacin and ionic currents through the single OmpF channel in the presence of 5 μM enrofloxacin added to the trans side at +100 mV. 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 interest.

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