

# Noise analysis of antibiotic permeation through bacterial channels

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## ABSTRACT

Statistical analysis of high-resolution current recordings from a single ion channel reconstituted into a planar lipid membrane allows us to study transport of antibiotics at the molecular detail. Working with the general bacterial porin, *OmpF*, we demonstrate that addition of zwitterionic  $\beta$ -lactam antibiotics to the membrane-bathing solution introduces transient interruptions in the small-ion current through the channel. Time-resolved measurements reveal that one antibiotic molecule blocks one of the monomers in the *OmpF* trimer for characteristic times from microseconds to hundreds of microseconds. Spectral noise analysis enables us to perform measurements over a wide range of changing parameters. In all cases studied, the residence time of an antibiotic molecule in the channel exceeds the estimated time for free diffusion by orders of magnitude. This demonstrates that, in analogy to substrate-specific channels that evolved to bind specific metabolite molecules, antibiotics have 'evolved' to be channel-specific. The charge distribution of an efficient antibiotic complements the charge distribution at the narrowest part of the bacterial porin. Interaction of these charges creates a zone of attraction inside the channel and compensates the penetrating molecule's entropy loss and desolvation energy. This facilitates antibiotic translocation through the narrowest part of the channel and accounts for higher antibiotic permeability rates.

**Keywords:** single molecules, antibiotic transport, channel reconstitution, lipid bilayers

## 1. INTRODUCTION

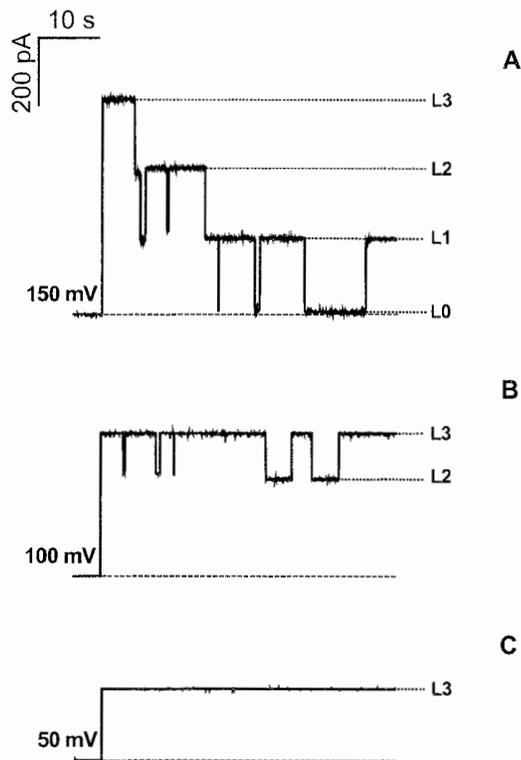
Recent advances in single-molecule studies<sup>1</sup> have unveiled new perspectives in physics and biology by providing a wealth of knowledge about interactions and dynamics in the nano-scale world. Together with the availability of structural information obtained with atomic resolution, this allows for better understanding of the molecular mechanisms responsible for basic cellular processes. Last year we have shown how such studies can aid rational design of drugs by characterizing the molecular details of antibiotic translocation through bacterial walls.<sup>2</sup> We have been able to resolve elementary events of single ampicillin molecule translocation through the channels formed by the general bacterial porin, *OmpF*.

It is well known that membrane permeability barriers are among the main reasons for bacterial antibiotic resistance.<sup>3</sup> Ampicillin and other  $\beta$ -lactam antibiotics act on the peptidoglycan layer, which is located between the outer and inner bacterial membranes and which protects bacterial cells mechanically. These antibiotics are structural analogues of the terminal D-ala-D-ala unit that participates in peptidoglycan synthesis. They inhibit peptidoglycan biosynthesis by binding to transpeptidase and eventually lead to bacterial death.

## 2. RESULTS AND DISCUSSION

The general diffusion porin *OmpF* (Outer membrane protein F) is the main pathway for  $\beta$ -lactam antibiotics to cross the bacterial outer membrane into the periplasmic space.<sup>3</sup> We reconstitute this protein in planar lipid membranes (for a brief description of the method, see the chapter by Kullman et al. in this volume) to study the ionic current flowing

through a single channel. *OmpF* channel reconstitutes as a trimer, which can be tested by applying transmembrane voltages that close the channel in three characteristic steps, as shown in Figure 1.



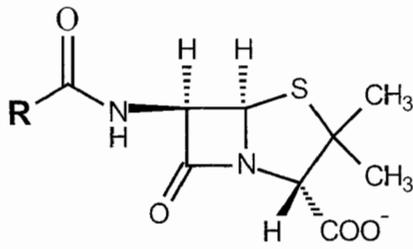
**FIGURE 1.** Typical recordings of ionic currents through single trimeric *OmpF* channels reconstituted into planar lipid bilayer membranes at 150 mV (A), 100 mV (B), and 50 mV (C) show that channel stability depends on the applied voltage.<sup>4</sup> Large voltages close the channel in three approximately equal steps revealing its trimeric organization. Dotted lines L3, L2, L1, and L0 correspond to fully open, one-monomer closed, two-monomer closed, and three-monomer closed states, correspondingly. The dashed line shows zero-current level. Membranes were formed from a 1% solution of diphytanoyl phosphatidylcholine. The membrane-bathing solution contained 1 M KCl, 1 mM CaCl<sub>2</sub>, and 5 mM HEPES at pH 7.0. Time resolution was 10 ms.

It is seen that the channel conformational stability depends on the applied voltage. At voltages of 100 mV and higher it ‘gates’ to partially or fully closed conformations on the time scale of several tens of seconds. However, on smaller time scales the channel is stable enough to serve as a molecular sensor for studies of molecule transport.<sup>5-8</sup> The underlying idea of molecular sensing is the same as in the resistive pulse technique that has been used in Coulter counters for nearly fifty years.<sup>9</sup> It can be formulated as follows. If a non-conducting particle suspended in a conducting medium moves into a small capillary, it decreases the conductance of the capillary relative to that of the capillary filled with the conducting medium alone. The magnitude of the decrease in conductance is related to the particle size; the duration of the decrease depends on the velocity of the particle in the capillary. In conventional Coulter counters the particle suspension is made to flow by a hydrostatic pressure difference maintained across the capillary, so that it is the flow velocity that defines the duration of the conductance decrease. In molecular sensing the leading processes are diffusion and interactions between a particle and a channel.<sup>8</sup>

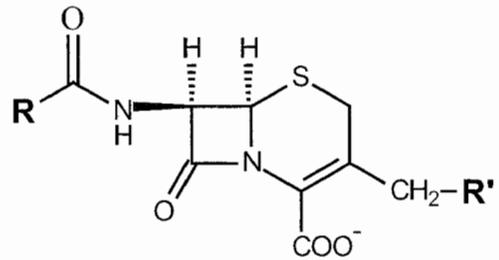
In the absence of particle/channel interactions, diffusional relaxation in molecular channels is impressively fast. Its characteristic time, calculated as  $L^2/12D$  (refs.<sup>10,11</sup>), where  $L \approx 5 \times 10^{-9}$  m is the channel length and  $D \approx 3 \times 10^{-10}$  m<sup>2</sup>/s is the diffusion coefficient of a nanometer-sized particle, is in the range of nanoseconds. However, because of interactions between a particle and a channel this time is often orders of magnitude larger.<sup>8</sup>

It is well known that substrate-specific channels such as sugar-specific *maltoporin* or *LamB*<sup>12</sup> have evolved to bind corresponding substrates in order to facilitate their transport into the cell. In fact, in the case of *LamB* this binding is so strong<sup>13</sup> that recently it has become possible to detect translocation of single sugar molecules<sup>14,15</sup> as time-resolved transients in the ionic current through the channels.

These two schemes illustrate the common chemical structure of  $\beta$ -lactam antibiotics:



Penicillins

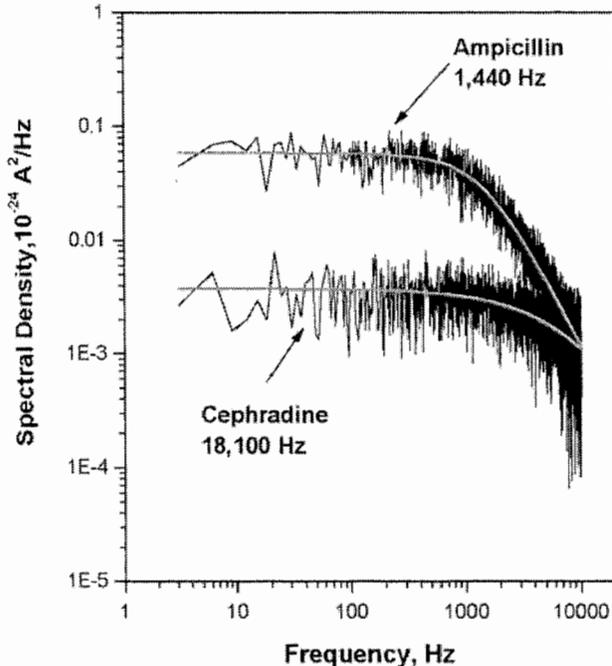


Cephalosporins

where **R** and **R'** designate variable chains of different size and charge.

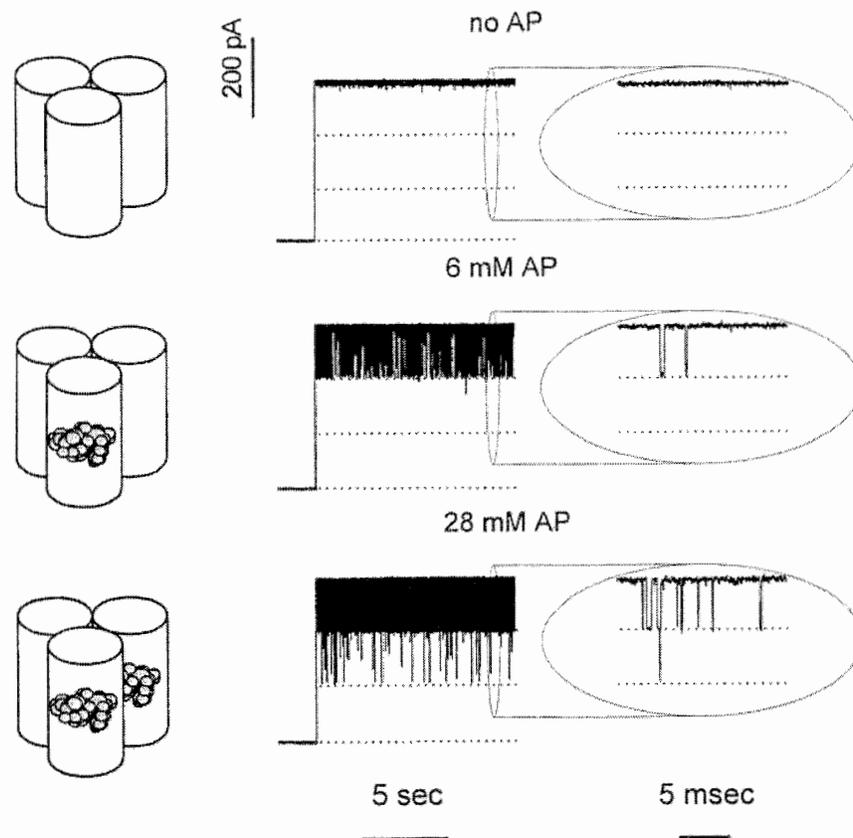
The questions we ask here are: Is the interaction between effectively penetrating drugs and the outer membrane channel strong enough to render the passage events resolvable? Is there a correlation between the antibiotic efficacy and the strength of this interaction?

Noise analysis of the ionic current through a single *OmpF* channel in the presence of effective  $\beta$ -lactam antibiotics reveals significant excess noise. Figure 2 shows the power spectra of this noise for two different antibiotics. These spectra are dominated by single Lorentzians of differing amplitude,  $S(0)$ , and corner frequency,  $f_c$ . Depending on the voltage, pH, and (most of all) antibiotic, the characteristic relaxation times, calculated from the spectra, differ by orders of magnitude varying from microseconds to hundreds of microseconds. In particular, for cephradine this time is  $1/(2\pi f_c) = 8.8 \mu\text{s}$ ; for ampicillin it is  $110 \mu\text{s}$ . These times should be compared with the several nanoseconds expected for purely diffusional relaxation of the molecules of this size.



**FIGURE 2.** Power spectral density of the excess noise of a single *OmpF* channel in the presence of two different  $\beta$ -lactam antibiotics, ampicillin and cephradine. Smooth solid lines are Lorentzians whose corner frequencies differ by a factor of more than ten. Antibiotics were added at 20 mM bulk concentration to 1M NaCl aqueous solution buffered by 5 mM HEPES at pH 7.4. The applied voltage was -100 mV; the membrane lipid composition was same as stated in Fig. 1 caption.

Thus, for some antibiotics, the characteristic time obtained from noise analysis is well within the range that is accessible for the time-resolved single-molecule measurements. Figure 3 shows results of such measurements for a penicillin drug, ampicillin.<sup>2</sup>

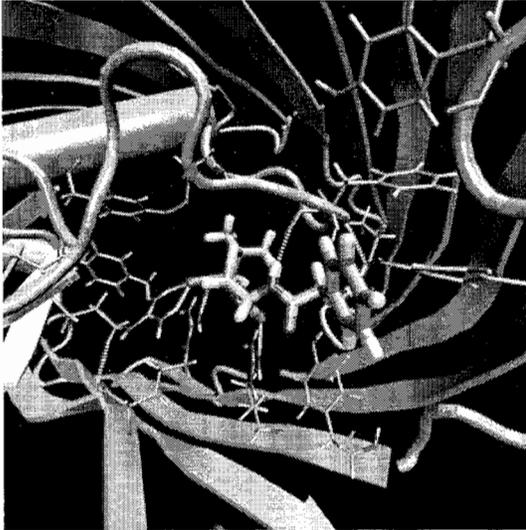


**FIGURE 3.** Penetrating ampicillin (AP) molecules modulate ion current through a single *OmpF* channel. Membrane bathing solution was 1M KCl, pH 6.0, the applied voltage was -100 mV. **Top:** In the absence of antibiotic the ion movement is mainly determined by the geometry and the surface properties of the channel pore. The ion current is stable, no interruptions are seen even at the high resolution recording shown on the right. **Middle:** In the presence of ampicillin in small concentrations one of the three *OmpF* pores gets spontaneously blocked by a translocating drug molecule. At the time resolution of 0.15 ms (left) blockage events look like downward spikes. At the higher resolution of 0.015 ms (right) they are seen as well-defined steps to 2/3 of the open channel current and back. Time between blockage events and their width provide both thermodynamic and kinetic parameters of antibiotic-pore interactions. **Bottom:** At higher ampicillin concentrations channel blockades are more frequent. Sometimes they overlap in time, leading to a transient reduction of ion current to 1/3 of its initial value.

To understand which structural features of  $\beta$ -lactam antibiotics determine their permeation properties through *OmpF* porin, we have simulated the ampicillin transport. Our model is based on the three-dimensional structure of the wild type *OmpF* monomer resolved at 2.4 Å (ref.<sup>16</sup>) that is immersed in a micelle-like environment formed by 100 molecules of LDAO detergent and hydrated by 7600 water molecules, 39 K<sup>+</sup> and 29 Cl<sup>-</sup> ions. The zwitterionic ampicillin was docked at the channel constriction as previously described.<sup>2</sup> The entire system of more than 33,000 atoms was simulated for 4 ns. A 10 ns non-equilibrium MD trajectory has been generated to allow ampicillin translocation through the pore. Then, using equilibrium MD we simulated a few characteristic ampicillin conformations found during the path for a total of 15 ns.<sup>17</sup>

It appears that the main chemical groups of the zwitterionic ampicillin interact with channel residues in the eyelet region (Figure 4). These interactions compensate the entropy loss and the desolvation energy, thus increasing the drug translocation probability. The contact points between the charged or polar groups of ampicillin and arginines on one wall and acid residues on the opposite wall are of electrostatic origin. The phenyl ampicillin group (at the R end

of the molecule) also gives a favorable energetic contribution during translocation by interacting with the hydrophobic environment. These simulations provide new tools to interpret results for other antibiotics.



**FIGURE 4.** A model for ampicillin docking in the narrowest part of the *OmpF* monomeric pore, top view. The zwitterionic ampicillin simultaneously interacts with the positively and negatively charged residues at the constriction zone formed by the  $\beta$ -barrel wall and loop L3. On one side the carboxylate ampicillin group is attracted to the cluster of positively charged residues in the pore, and on the opposite side the ammonium ampicillin group is attracted to the carboxylate of Glu-117. The skeleton of *OmpF* is shown in a ribbon representation. Both the key residues inside the pore and the ampicillin molecule are shown using stick representation, but the drug is represented by bulkier sticks.

We find that:

- The zwitterionic form facilitates drug translocation.
- For the penicillins, addition of a hydroxyl group at the **R** extremity leads to a shorter residence time. This effect clearly can be attributed to a loss of hydrophobicity at this part of the drug.
- The lower binding strength for cephalosporins compared to their respective penicillin analogues can be explained by changes in the charge distribution induced by the  $sp_2$  carbon atom, which carries the carboxylate group.

#### 4. CONCLUSIONS

This work demonstrates that single-molecule measurements combined with noise analysis and molecular dynamic simulations based on high-resolution X-ray protein structure are able to clarify the mechanisms of enzymatic activity on the atomic scale. We expect that these kinds of studies will have multiple applications for directed drug design in the near future.

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