[Frontiers in Bioscience 14, 3222-3238, January 1, 2009]

Simulating transport properties through bacterial channels

Matteo Ceccarelli

Dipartimento di Fisica and SLACS-INFM, Universita degli Studi di Cagliari, Cittadella Universitaria di Monserrato, SP Monserrato-Sestu Km 0.700, 09042 Monserrato, Italy

TABLE OF CONTENTS

1. ABSTRACT

Gram-negative bacteria have several porins in their outer membrane: they act as gates in the exchange of molecules and are also considered the main pathway for antibiotics. Bacteria are able to resist the action of antibiotics simply by closing off physical access to their interior, either underexpressing porins or decreasing the porin's internal size. The dissemination of pathogens resistant to common antibiotics requires the development of new classes of drugs with improved properties. Understanding how compounds diffuse through bacterial porins can aid in the design of new antibiotics with better penetration power, partially solving one of the main problem of resistance. The diffusion of antibiotics through porins is a molecular-based process, controlled mainly by electrostatic interactions, as has been shown experimentally. Data from single molecule experiments are available in the literature but provide only indirect evidence of the transport. Molecular dynamics simulations at the molecular scale, combined with a recent algorithm able to extend simulations to biologically interesting times, have been shown to complement experiments, providing detailed information on the transport of antibiotics through porins.

2. INTRODUCTION

In the second half of the last century, the problem of infectious diseases caused by bacteria appeared to be closed. With hundreds of new antibacterial drugs commercialized, pharmaceutical industries demonstrated an ability to produce life-saving drugs in a timely manner. While society benefited from this success, at the same time it became more difficult to place new antibiotics in the market. Due to a low expected income stream, the majority of pharmaceutical companies have preferred to invest money in other fields, for example where drugs are prescribed for chronic diseases. Investment and research in antibacterial compounds slowed, and the number of new antibiotics registered has decreased to only few units in the last decade (1,2).

The demand for new drugs is related to the problem of bacterial resistance, i.e., a decrease in efficacy of an antibiotic in clinical use with respect to laboratory tests. It is worth noting that each new antibiotic has encountered resistance, sometimes even before its commercialization. However, bacterial strains resistant to antibiotics have become a serious problem, especially in

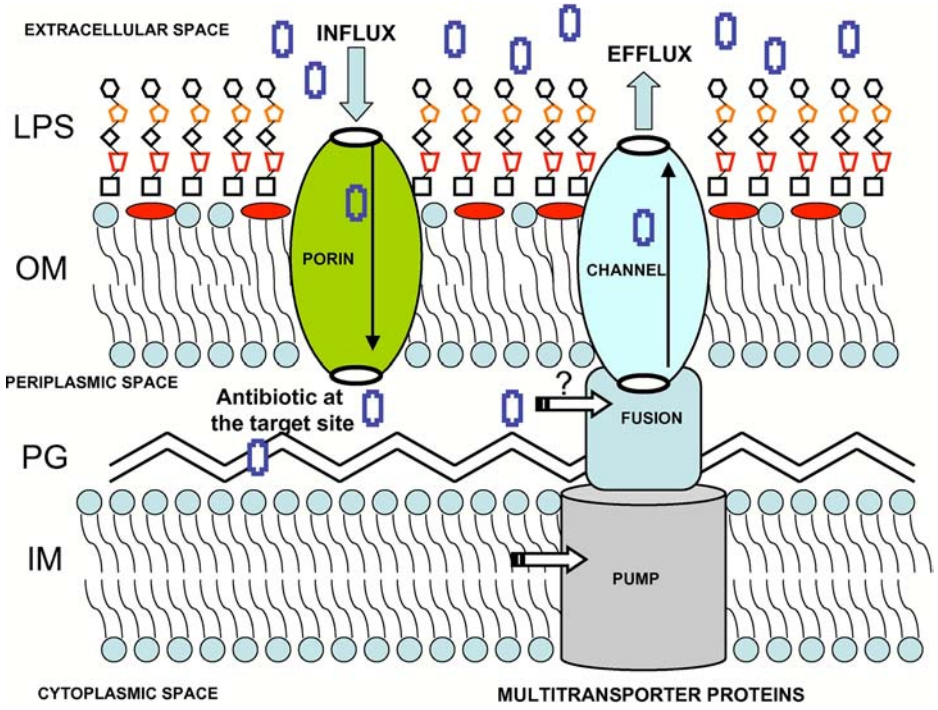


Figure 1. Schematic representation of both the inner and outer membrane of Gram-negative bacteria together with the porous layer of peptidoglycan, the main target of beta-lactam antibiotics (in blue). Influx and efflux systems are also inserted to show the uptake and suggested extrusion of antibiotics.

hospitals. Bacteria have developed many mechanisms to resist antibiotics, and sometimes more than one can act simultaneously. While on one hand the few new compounds are mainly modifications of existing classes of compounds, phatogens have developed multidrug-resistance, i.e., resistance to more than one class of antibiotics. There are many factors that lead to this scenario, ranging from a poor use of antibiotics (over-use and under-dosage or an incomplete treatment courses) to an increased transmission of diseases due to urbanisation and globalization (3,4). Nowadays, the main challenge for pharmaceutical industries is to discover new antibiotics. However, only a new class of antibiotics with improved properties would have the potential to combat emerging and re-emerging pathogens that have developed different defence mechanisms. Since the antibiotics that were easy to discover have already been found, a new class of antibiotics will require major efforts and many resources (5,6). Given the different mechanisms of resistance to antibiotics and the different processes controlling them, a multidisciplinary approach seems to be the only way to tackle this problem in a rational and perhaps less expensive way (7).

The path leading to the next generation of antibiotics passes through the knowledge of the resistance mechanisms and the molecular processes governing them. Some defence mechanisms are based on chemistry. By recognition of the antibiotic as an external and toxic compound, bacteria can alter the molecule in different ways. Some proteins are able to either modify the antibiotic's covalent bonds or to add new chemical groups which inhibit binding (8,9). The same results can be obtained by modifying the target site, even if this mechanism is more sensitive and subtle, since it could change the functionality of the target itself. Other defence mechanisms rely on the perturbation of diffusion properties. Antibiotics, as with any drug whose target is internal, need to penetrate to exploit their antibacterial activity. Bacteria are protected from external toxic compounds by an internal cell membrane (IM) and an additional outer membrane (OM, in gram-negative bacteria). Among the different factors leading to resistance of bacteria to antibiotics, the simplest one is decreased access to target site (10,11). Focusing on the gram-negative bacteria, where the outer membrane represents a true barrier for any hydrophilic compound, this mechanism is controlled by two groups of membrane proteins (see Figure 1). The former control the influx and the latter the efflux of compounds through the outer membrane, and bacteria can act on the expression of both proteins to diminish internal concentration of antibiotics.

There are many differences between the influx and efflux systems. Influx is controlled by porins, water-filled channels located in the outer membrane (12). The uptake of antibiotics is due to a concentration gradient: supposing the internal concentration is always close to zero (the antibiotic once inside binds the target), antibiotics penetrate by passive diffusion. Efflux systems are more complex, with a multitransporter protein spanning both the inner and outer membranes (see Figure 1). These multitransporter proteins recognise the toxic compound, bind it, and then efflux against the gradient by using energy. The structure of efflux proteins as well their

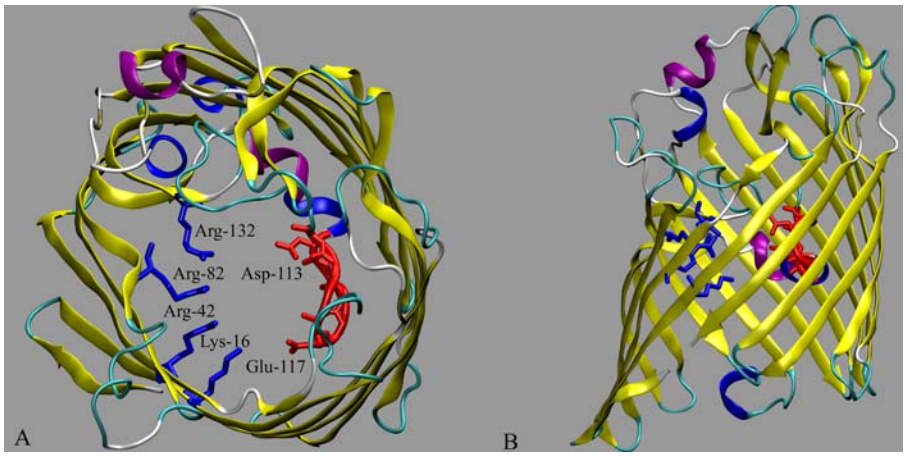


Figure 2. The X-ray three-dimensional structure of OmpF porin with highlighted the charged residues of the constriction region (Arg-42-82-132, Lys-16 in blue and Glu-117, Asp-113 in red) as viewed from the extracellular side.

mechanism is not well known in all its details and is still under debate (13,14,15) Bacteria can control the uptake of small molecules, becoming resistant either by underexpressing some general diffusion porins in OM or mutating some key amino acids of those porins (16,17). Alternatively, they can act on the efflux system by overexpressing multitransporter proteins (18).

This review focuses on the process that controls the influx of antibiotics through the outer membrane. The same approach can be used for the efflux, relative to the process of extrusion of the molecule. The physics community can provide some answers to such a problem, which is based on passive diffusion at the molecular scale. As shown by experiments, this diffusion is controlled by electrostatic properties, i.e., molecular properties of the antibiotics and the channels involved (19). Moreover, simple analytical models, even with a rather crude modelling of molecule/channel interactions, have pointed out how interactions are important to tune the flux of particles traversing the channels (20,21,22). Then, simulations can be of great help at this level to set up a model for antibiotics transport. However, standard molecular dynamics simulations cannot approach the time scale on the order of the diffusion of antibiotics through porins, which is hundred of microseconds. To solve this time scale problem, there are now different algorithms proposed to accelerate the simulated processes that can occur in the time manageable by simulations, which is tens of nanoseconds. As discussed here, simulations provide a good complement to experiments, providing detailed information that could be useful to design new antibiotics with improved transport properties (7,23).

This review is organized as follows: the first sections are devoted to describe the systems involved in this research, background on porins and antibiotics, and finally, the methodologies and experiments with findings and simulation techniques. The last part presents the results from all-atom MD simulations. The paper ends with conclusions and perspectives.

3. THE ACTORS: PORINS AND ANTIBIOTICS

The biological world is based on a compartmentalization of specialised environments (24). Cells and bacteria are protected by an external barrier that defines the inside and the outside and prevents the uptake/leaking out of external agents/molecules. Moreover, in the course of evolution, these organisms developed other internal subregions with focused tasks, such as mitochondria, chloroplasts, peroxisomes, and lysosomes. This organization requires a dividing element with main functions to allow the exchange of materials (molecules and ions) and information (either through specific molecules or through a change in physical properties). This complex system is the biological membrane, a sheet-like structure composed of lipids with a thickness between 60 and 100 Å, which make it impermeable to all molecules but those which are hydrophobic; it also contains membrane proteins, which perform all the specialised tasks, such as enhancing the flux of hydrophilic molecules and ions. These proteins interact strongly with the membrane, and are either fused inside or located on its surface, such that the membranes are dynamic structures in which proteins float in a sea of lipids (24).

A unique classification of membrane proteins does not exist, reflecting the high variety in this protein family. For the purposes of this paper, the focus is on channels where the transport process relies on passive diffusion driven by a gradient concentration. These channels can be divided into two categories depending on their facility for transporting small molecules: selective vs. non-selective. While selective proteins are highly specialized structures devoted to transporting only one species, such as maltoporin with sugars (25,26,27,28,29), non-selective membrane channels facilitate the permeation of small solutes, ions, and water (11). In among these channels there are porins, which are beta-barrel proteins with 16 and 18 barrels (trimeric form) or 12 and 14 barrels (monomeric form). Their structure is cylinder-like with a water-filled pore spanning the entire membrane. Examples of such proteins in bacteria are OmpF, OmpC, and PhoE. The most studied one is OmpF from *E. Coli* (see Figure 2), a trimeric porin with 16 beta-barrels that also shows a small

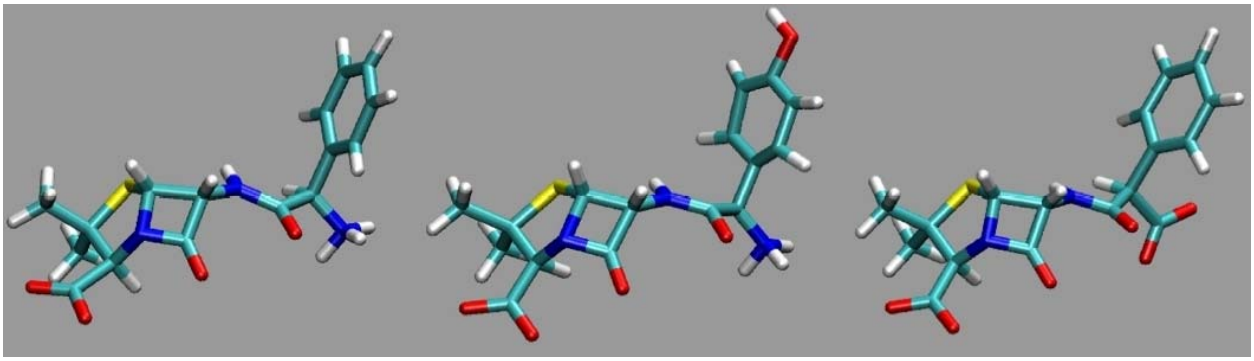


Figure 3. From the left to the right: Ampicillin, Amoxicillin, and Carbenicillin, three antibiotics of the Beta-lactam family. The central four-members ring is the active part that binds to the bacterial target, the peptidoglycan.

cation-selectivity, with each monomer charged at -10 at neutral pH.

In bacteria, general diffusion porins are used to uptake nutrients or expulse waste products (12). The OmpF is also considered one of the main entry points for different classes of antibiotics to pass through the outer membrane (10, 30). The high-resolution crystal structure (31) unveiled its structural features. On overall, the stability of the trimer relies on the loop L2 of each monomer that makes contacts with the adjacent subunits. Another loop, the L3, gives to OmpF the characteristic internal shape of a double-funnel. Contrarily to all the other short loops that point outwards, it folds inside creating a narrow region at mid-height. This region, called the constriction region, is supposed to be the bottleneck for transport properties. The presence of the loop L3 prevents the passage of large molecules through the channel, whose sizes are roughly 7x11 Å, as can be obtained from inspection of its X-ray structure and simulations (32,33). This value agrees well with the supposed 12 Å diameter, obtained by investigating sugar transport with different size molecules (34). The other characteristic of the constriction region is the presence of many charged amino acids. On one hand there are three arginines, R42-82-132, facing L3. On the other hand, the loop L3 has two negatively charged amino acids, D113 and E117, whose lateral chains point toward the three arginines together with the carbonyl groups of the other amino acids, (see Figure 2), overall creating a high transversal electric field (35). The main question points to the role of loop L3 in gating molecules through the control of the size of the pore and the electrostatic properties.

Beta-lactams, together with fluoroquinolones, can use OmpF to overcome the OM of bacteria. Indeed, some Beta-lactam resistant strains of *E.Coli* have shown a deficient OmpF production (10) and/or point mutations at the level of the OmpF's constriction region (36). These antibiotics are characterized by a four-member ring (see Figure 3), a natural group that binds peptidoglycan, a highly porous layer located between the outer and inner membrane of gram-negative bacteria. Among Beta-lactams, three common antibiotics belonging to the subclasses of penicillins have a size that fits the OmpF central region and have been considered here: ampicillin, amoxicillin, and carbenicillin (see Figure 3). While their sizes are similar, they differ in physical properties. Amoxicillin and ampicillin are zwitterionic, compared to carbenicillin which is anionic with a total charge of -2. Amoxicillin, with an additional OH group attached to the phenyl ring, is more hydrophilic with respect to the other two. These small differences are enough to provide experimental differences in their behaviour during translocation through OmpF(19).

4. EXPERIMENTAL FINDINGS AND MODELLING

4.1. Single-molecule experiments

Knowledge of molecular transport through porins comes from experiments conducted on a large ensemble of porins incorporated into planar lipid bilayers (37) (Black Lipid Membrane experiments) or in liposomes (38,39,40,41), although the latter are difficult to reproduce. The BLM technique is based on the formation of a lipid bilayer over a hole separating two chambers. Each chamber is filled with a buffered ionic solution and contains an electrode used to maintain a constant potential. As channels are inserted in the membrane and an external voltage is applied, ions start to flow from one side to the other, producing a net current that depends on the applied electrostatic potential. Each channel has a typical current that can be used as an electrophysiological fingerprint. Molecules inserted into the chambers modify the ion current via an interaction with the channels. Information about the rate constant, related to the number of molecule per second interacting with the channels, can be obtained using the analysis of molecule-induced current noise with the results extracted from frequency spectra (42).

Multichannel experiments are very attractive for a quantitative description of the molecule/channel interactions due to better statistics. However, dynamical properties are hidden in averaged ensemble measurements. Recent years have seen significant advances in single molecule detection techniques, allowing the study of behaviour of individual molecules in real-time (43,44), (see Figure 4, a pictorial representation of the experimental device). Single-molecule experiments on reconstituted porins in BLM have indirectly measured the translocation of sugar molecules by measuring the associated ion current fluctuations (20,29,44,45). In particular, when working

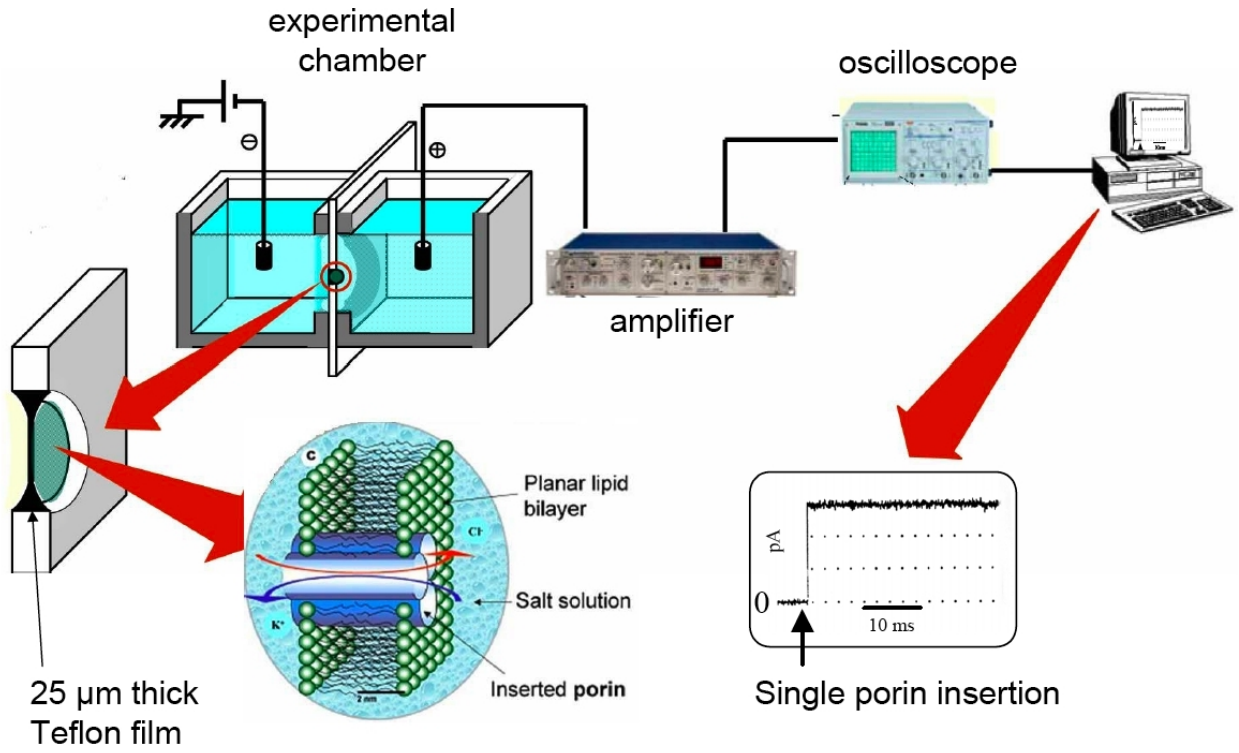
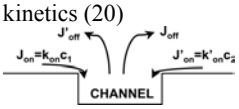


Figure 4. Scheme of the device for single molecule experiments on reconstructed channels in a BLM. Obtained from Mathias Winterhalter's group at Jacobs University, Bremen, DE.

with trimeric channels, ion currents show stepwise transitions once the solute concentration is increased or other parameters are adjusted (buffer solution, pH). These experiments showed that the current has exactly four values, from 1 (all monomers conducting) to 0 (all monomers closed) with steps of 1/3 (see Fig.5). It turns out that molecules do not simply perturb the current but they completely block the passage of ions in each individual monomer; the total current is the sum of the contributions of each individual monomer which can be either conducting or not conducting. In general, the occupation time is on the order of hundreds of microseconds and can be solved with commercial amplifiers. The two important quantities measured are the number of blockages and the average time of blockages. Molecule/channel interactions can be investigated by measuring current properties as a function of parameters such as pH, buffer solution, applied external voltage, and solute concentration. Due to the limited size of the channel, a single occupancy is always supposed.

4.2. Modeling translocations from blockages

In single-molecule experiments, an interruption of the current on the order of hundreds of microseconds means a strong molecule/channel interaction; it does not directly mean translocation, but simply that a molecule occupies the channel. However, it turns out that the occupation occurs when solute is added either on the cis or trans side (the cis side is the chamber where the channel is added), with the same blockage time. This means that the binding site is unique and accessible from each side of the channel. In the simplest case, one can describe the occupation site as an energetically binding site accessible from both sides and the process can be solved by chemical kinetics (20)



where the incoming fluxes are products of the solute c_i and rate constants k_{on}. The current shows a transition (see Figure 5) when a molecule is captured by the binding site. Once captured, the molecule can either go back, with no net translocation, or can continue to diffuse in the same direction, which corresponds to translocation. Following this simple binding-site model (20), as upper limit, for every two blockages of the current there is one translocation; experimental blockages are correlated to the flux of molecules through channels. However, the open question is what can be gained from these experiments? Analytical models can provide an answer.

In the last few years, more sophisticated models to investigate the problem of molecules translocation have appeared in literature. Starting from the binding-site model, one can obtain that when the concentration is zero on one side, the flux J is

$$J = \frac{k_{on} c_1}{2[1 + k_{on} c_1 \tau]} \quad (1)$$

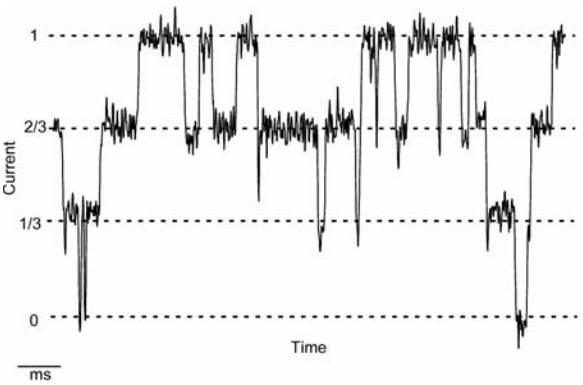


Figure 5. Typical behaviour of ionic current as function of time in a trimeric channel after addition of solute. Each current transition correspond to a monomer blocked by a solute molecule. Closure of more than one monomer can occur.

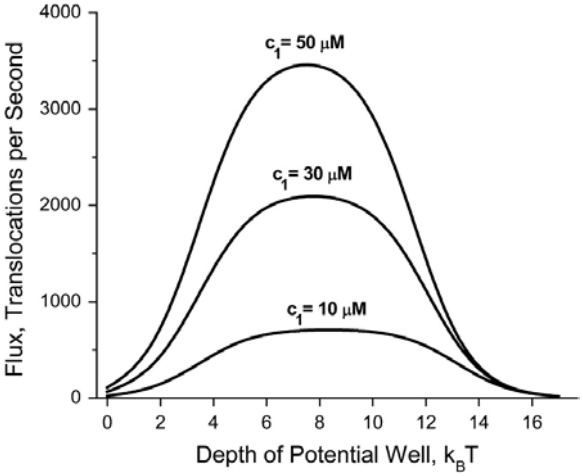


Figure 6. Nonmonotonic behaviour of the flux of molecules vs. the binding strength at different solute concentration as obtained from the diffusion model of Ref. 21.

where k_{on} is the rate of molecules arriving at the mouth of the channel, c_1 is the concentration, and τ is the binding strength of the channel. It turns out that when the binding site is more attractive (τ larger), the flux decreases: the presence of the binding site seems to decrease the flux. This statement is in contrast with the fact that for selective channels, one would expect a high flux when the concentration is high **(**46,47). A selective channel described as a simple binding-site for translocating molecules does not seem appropriate.

Focusing on the example of the selective bacterial porin LamB, the existence of an extended binding zone for oligosaccharides is firmly established (27,34). Berezhkovskii and Bezrukov proposed a diffusion model for the motion of molecules in the channel (21). This model can be represented by the same kinetic scheme of the above binding-site model (20). The main difference resides in the way the authors treat the molecule/channel interactions. While the molecule sees the channel as a square-well potential U that occupies the whole length L, as in the binding-site model, the translocation is seen as a slow diffusion process with a position-dependent diffusion constant. Describing the propagation of the molecule by Green's function and solving the Smoluchowski equation, one obtains the flux as a function of channel and molecule parameters. Contrary to the previous model, the flux is not a monotonic function of the binding strength; for each concentration there is an optimal depth of the binding site that maximizes the net flux, as reported in Figure 6. Importantly, the predicted rates are of the same order of magnitude as those obtained experimentally (48).

Another point touched on by analytical models is the asymmetry of the flux. It turns out that microscopic processes are time reversible. Time-reversal symmetry has been used explicitly in defining an algorithm to find reactive paths, the transition path sampling (49,50,51,52). This reversibility in time applies also to molecules translocating through channels. Even modelling the molecule/channel interactions as a downhill potential, the resulting flux is symmetric (53). In order to break this symmetry and obtain a ratchet-like system, some authors introduced non-equilibrium fluctuations. In the case of passive diffusion of glycerol, the presence of an asymmetric potential combined with non-equilibrium fluctuations makes the flux asymmetric (54). The latter condition is attractive since the transport of molecules does not strictly follow the gradient concentration. The question remains as to where the non-equilibrium fluctutations come from.

Recently, two other papers using different translocation models pointed out an asymmetric flux without introducing non-equilibrium fluctuations. For long channels which can be blocked by one molecule, it is important to note where the binding site is located with respect to the entry and exit points (22). Intuitively, once the single molecule is bound, its escape is faster to the closest side, producing an asymmetry in the flux. Even with a stochastic model of a channel, considered as N internal binding sites for molecules which are translocating, it is possible to obtain an asymmetric flux by introducing a strong interaction site at different positions along the channel (55). Again, the shape of the underlying potential controls the efficiency of the transport, with binding sites producing the most efficient transport for small external concentrations, and repulsive binding sites more suitable for large concentrations. All of these simple models strongly indicate how the molecule/channel interactions are important in determining the flux. Or, reversing the point of view, it is possible to tune the flux by changing the molecule/channel interactions. The solution to the inverse problem is the basis for the design of powerful antibiotics by utilizing ad hoc molecular transport properties that enhance their flux through porins.

4.3 Antibiotics vs. sugar transport

The above analysis suggests that analytical models are very useful for gaining insights into transport properties of molecules through channels and can help to

interpret experiments. Focusing on sugar transport through specific channels, single-molecule experiments (48) and the diffusion model (21) agree that, roughly, at micromolar concentration, the number of blockages is on the order of a few thousands. Following that model, the process can be seen as the slow diffusion of a molecule interacting with the channel wall via aromatic residues, and it has been labelled as greasy slide (27). Moreover, the specificity of the channel is seen in the model through the non-monotonic behavior of the flux as a function of the concentration (21). One can assume that for sugar transport there is a consensus on the way molecules are transported. Conversely, the field of antibiotics transport through channels is almost new and a consensus is far from being reached.

The uptake of antibiotics naturally does not occur through specific channels but through general diffusion porins such as OmpF. Looking at single-molecule experiments, one can see a remarkable difference in the number of blockages. At millimolar concentrations, the number of blockages with antibiotics is a few tenths (19). For sugars there are few thousand blockages at micromolar, and extrapolating at millimolar one obtains few million blockages (21,48). The decrease in the net flux of antibiotics through general channels with respect to sugars through specific channels is around 10^5. By using a simple analytical model, this difference can be taken into account either by modifying the internal potential with additional barriers, or by decreasing k_{on}. However, this is the simplest and fastest way to find a solution, while a new model calibrated on experimental and theoretical data might help to develop a theory for antibiotics transport. The ultimate goal is to yield general criteria that can positively bias drug design.

4.4. Antibiotics translocation is a molecular process

Single-molecule experiments on antibiotic transport through general diffusion porins pointed out that the translocation process is a molecular-based process that depends crucially on electrostatic interactions. The two quantities that are important in experiments are the number of blockages and the average blockage time. Experiments performed with ampicillin and OmpF showed clearly that the net flux depends on the electrolyte concentration and on the pH of the solution (19). When the electrolyte concentration decreases, the electrostatic interactions increase their range of action and the net flux increases as well. On the contrary, when the electrolyte concentration increases, and in the solution charged groups are better screened by ions, a decrease of the flux occurs. This is a signature of the electrostatic nature of the process, since molecules are more attracted toward the constriction region of OmpF when electrostatic screening is low. The effect of screening on the blockage time is not as large as for the flux, showing that the blockage time is more affected by local interactions. This is also evident from the pH dependence. Ampicillin is zwitterionic at pH 5. When the pH changes, there is a large effect on the number of events that decrease dramatically. At the moment, this behaviour is not clear, and a possible speculation is that pH affects also the charged groups of OmpF. It is worth noting that the central region of OmpF is rich in charged groups (see Figure 2), and in particular the transversal electrostatic field could help zwitterionic antibiotics to arrive near the central region. When this condition is not satisfied, for example, by changing the pH which would transform ampicillin in a charged molecule, the process is higly affected.

The potential profile used in analytical models (21,22,55) is rather crude, but with the availability of modelling, it would be possible to improve knowledge of the antibiotic-channel interactions at the molecular scale. Moreover, it is important to point out that experiments indirectly measure the translocation and that the device has a resolution of 10-20 microseconds. When no blockage is seen, one cannot conclude that antibiotics are not translocating. It would be possible to have translocation without blockages because (i) either the molecule resides in the channel for less than the resolution time, or (ii) it does not completely occupy the pore channel. In principle, these issues can be afforded and solved using molecular dynamics (MD) simulations. However, when applied to biological systems, one has to deal with the so-called time-scale problem: the typical times of biological processes exceed the time one can reach with standard MD simulations (~ 100 ns). In recent years, many techniques were developed to solve the time-scale problem and still use MD simulations to investigate biological processes. The next section is devoted to describe MD simulations and the metadynamics algorithm that, by accelerating simulations, gives the ability to probe the transport of antibiotics through OmpF while maintaining an all-atom description (23,56).

5. THE SIMULATION TECHNIQUES

5.1. Molecular dynamics

Molecular dynamics simulations constitute a very powerful method for investigating processes at the microscopic scale (57). By solving Newton's equation of motion with an iterative procedure, they are useful to investigate:

- Interactions at the molecular scale
- Structure determination
- Dynamical properties, or the effects of spontaneous fluctuations due to temperature.

They offer a very high resolution both in space (angstrom) and time (femtoseconds). However, these features become a problem when dealing with a complex system, such as in the life sciences. There, systems are characterized by supramolecular aggregation of large objects with complex interactions, and many degrees of freedom with multicenter interactions mediated by solvent properties. This results in processes controlled by a multitude of weak interactions acting on a multiscale time, from microsecond to second. In particular, simulations at the molecular scale fail for two reasons: (i) systems with a too high number of atoms, and (ii) typical timescale order of magnitude larger than the integration step of Newton's equation of motion (fs) (58). In our particular case, the

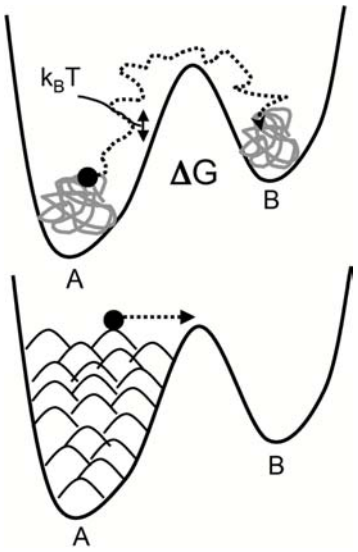


Figure 7. Scheme of a double well separated by a barrier ΔG higher than the average kinetic energy k_BT. Top: sampling with standard MD simulations. Bottom: sampling with the accelerated metadynamics. In the first case, the reactive trajectory overcoming the barrier has a low probability to occur and is called a rare event.

typical times are in milliseconds, so that in femtoseconds, this means a number of integration steps of the order of 10^{12}. Even using the fastest CPU available and a program that can exploit parallelism, the lower limit for observing one event is around one year. This is the time scale problem.

5.2. Calculating transport properties

Following statistical mechanics, properties at the microscopic scale can be obtained by averaging over many sampled conformers compatible with some constraints, such as energy, temperature, volume, etc. While structural properties involve static averaging, transport properties involve the average of quantities at different times via a correlation function (59). This difference makes transport properties more affected by statistical uncertainty, or that longer simulations are needed to have smaller errors. For example, diffusion constants can be obtained easily for homogeneous liquids such as water. In this case, the statistic is enhanced by the fact that particles are indistinguishable and the average is extended over all particles of the simulation box (in general, several hundreds). When the diffusion constant refers to a single molecule in liquid water, in principle, one needs to simulate for a time that is hundreds of times longer in order to have the same accuracy (57). More appropriate methods exist to calculate transmission rates in case of inhomogeneous systems, such as for example the diffusion of molecules through a lipid bilayer. In this case a combination of the local information from the free-energy landscape and the diffusion model are used to obtain the permeation rate (59).

Another example is given by transport properties controlled by high barriers that must be overcome. Consider, as in Figure 7, a system with two main minima separated by a high barrier (Delta G), compared to the average thermal energy (k_BT). The average time for a simulation to observe the system overcoming the barrier (τ) can be extracted by the transition state theory:

$$k = \frac{1}{\tau} = \nu \cdot \exp\left(-\frac{\Delta G}{k_B T}\right) \quad (2)$$

where ni is the prefactor, that in case of molecular processes can be approximated by 1 ps^{-1}. When a simulation is started in the basin A, if the barrier is around 8 Kcal/mol, the simulation time to overcome the barrier is on the order of microseconds, while a barrier of 12 Kcal/mol corresponds to milliseconds. With standard MD simulations, a long time is spent in basin A before overcoming the barrier, a process defined as a rare event for simulations (61,62).

In biological systems, both situations, many small barriers (or a diffusive problem) and high barriers, contribute to create the time-scale problem with simulations, and special algorithms are necessary to deal with this condition.

5.3. Metadynamics

Standard MD simulations cannot afford the typical biological time scale without important approximations in the system. Either the system is described at a lower level, a coarse-grained description, or one can approximate the interaction energies, producing systematic errors. In the case of antibiotics transport, experiments showed that the process depends crucially on electrostatic interactions and on single charged groups (19). Consistently, the system must be described at an all-atom level with the exact long-range electrostatic contributions.

The other way to accelerate simulations is then to use a technique that overcomes the time scale problem by working on the MD, i.e., the evolution of the coordinates. Among the different methods proposed, the metadynamics (63,64) seems extremely powerful, as shown by its recent applications to biological processes (65,66,67,68). This algorithm requires that the process under investigation can be represented using a small set of reaction coordinates to be defined a priori and be process-dependent. When the slow evolution of the process can be attributed principally to these few coordinates, the artifice is to use an algorithm to accelerate the time evolution of these coordinates.

As described in the previous section, during MD simulations many conformations of the same free-energy minimum are sampled (see Figure 7). The MD technique is based on the Markov-chain scheme: the new conformer visited depends only on the previous one, or a small number of previously visited conformers. It turns out that the same conformer can be visited many times, i.e., memory effects are absent (61). The idea of metadynamics is to take advantage of the previous visited conformers, moving from an algorithm with zero-memory effects to a history-dependent algorithm, so-called non-Markovian dynamics. Within metadynamics, only the defined

coordinates are forced to evolve with a non-Markovian dynamics, maintaining all the others near equilibrium. Memory effects are taken into account through a bias on the potential energy: with a given frequency during the simulation, penalty terms are added in order to avoid, or to penalize, if the same conformer is visited many times (see Figure 7).

The core of metadynamics is to find a priori the few variables that better describe the process under investigation. These variables, called also collective variables, are complex functions of selected degrees of freedom, such as a geometrical coordinate (distances, angles, dihedrals) or an interactions-based coordinate, such as the coordination number of a group of atoms, the number of hydrogen bonds, or hydrophobic contacts between different groups. The few collective variables represent the reduced space where memory effects are introduced to accelerate their evolution. These variables, subject to the solely underlying empirical potential, would evolve with a long time scale, in general, with the typical time scale of the process. With metadynamics it is possible, in the range of a typical simulation (tens of nanoseconds), to observe a process whose time scale is several microseconds.

The first advantage of metadynamics is the capability to accelerate events. However, the choice of the reaction coordinates represents implicitly a way to test whether that process can be described by those reaction coordinates, or implicitly it is assumed as a mechanism for that process. In the case where they fail, any evolution of the system can be observed, and one is driven to find more appropriate coordinates, or to assume a different mechanism for that process. This procedure is implicitly a speculation on the mechanism governing the process.

Another advantage of this algorithm is the possibility to quantify the process through a free-energy description (64). In the metadynamics scheme, the Hamiltonian with the bias added in the reduced space of coordinates r=r(x) reads:

$$\tilde{H}(\mathbf{x},\mathbf{p}) = H(\mathbf{x},\mathbf{p}) + h_i(r) \qquad (3)$$

where **x** and **p** are the positions and the momenta of all the atoms of the system of interest and h is the "history-dependent" biasing potential. These repulsive functions fill the minima of the underlying free energy and, after a long simulation, tend to compensate exactly for the free energy, which, in turn, can be approximated by their sum (see Figure 7). If Gaussians are used as repulsive functions for the potential, the bias reads:

$$h_i(r) = \sum_i w_i \exp\left[-\sum_{k=1}^{N} \frac{\left(r_k(\mathbf{x},t) - r_k(\mathbf{x}_i,t_i)\right)^2}{\Delta r_k} \right] \qquad (4)$$

where w_i and Delta r_k are the height of the repulsive potential, and the scale factor for the k-th coordinate, respectively. The outer summation (index i) is over the time steps. The scale factor defines the range of action of the repulsive potential and represents a sort of resolution of the reconstructed free energy surface.

5.4. Comparing metadynamics

Since its development, metadynamics has been compared to umbrella sampling (69,70,71), another popular method used to quantify a process via the free energy reconstruction. It has been defined as a dynamical umbrella sampling where both define a reduced space; the reaction coordinates define where to add a bias, with the difference that in metadynamics the bias depends on time, while in umbrella sampling, the reaction coordinates are restrained around some fixed values, a sort of grid decompositon of the reduced space:

$$h_i(r) = \frac{1}{2} k\left(r(x) - r_i\right)^2 \qquad (5)$$

where the index i now runs over the predefined grid points, not the time as in metadynamics. In umbrella sampling, one performs many uncorrelated simulations, one for each point of the grid, with an equilibrium procedure. In this way, the possibility of following a mechanism on the fly is completely lost, as the procedure performs a simple integration in the reduced space. Using metadynamics, on the other hand, one has access to the time (metatime) evolution of the process needed to exit from minima and reach the saddle point of the free energy surface. The implementation of metadynamics which makes use of multiple walkers or parallel bias exchanges (72,73), different simulations that independently fill the free energy minima, is faster but loses the time evolution of the process.

The main advantage of metadynamics with respect to umbrella sampling is that it is not required to define the range of variation of the reaction coordinates a priori or the predefined grid, instead letting the system evolve toward the lowest transition state, thus obtaining the minimum free-energy landscape along the path connecting the two minima. This prevents sampling of uninteresting regions and, in principle, allows the introduction of a high number of reaction coordinates (68). However, the use of a time-dependent biasing potential is in some way a non-equilibrium procedure with respect to the other degrees of freedom, especially to the so-called slow modes. When the latter are not included in the chosen set of reaction coordinates, the choice of the parameters controlling the repulsive potentials (i.e., deposition time step, height, and scale factor), it is crucial to allow the system to equilibrate each time a new term is added. The efficient sampling of non-explicit slow modes within metadynamics can be tackled in different ways, either by improving the sampling with the replica exchange method (74) or by correcting the reconstructed free energy surface with a subsequent refining umbrella sampling (75). In some way, with metadynamics one solves the problem of high barriers. However, as stated above, the time scale problem in biological processes comes also from the presence of many small barriers, or a diffusive mechanism. The two methods, proposed to obtain a more realistic free energy

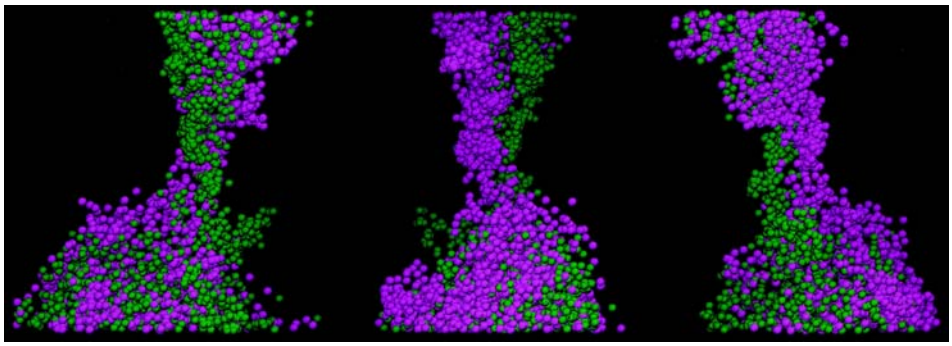


Figure 8. Superimposition of several frames from MD simulations of OmpF with 1M ion concentration (protein not shown). Ions follow different left-handed screw-like paths from extracellular (top) to periplasmic space (bottom) driven by charged residues. Reproduced with permission from Ref 84.

reconstruction within metadynamics simulations, account for the error due to the complexity of the landscape.

Another common methodology used to accelerate simulations via a biasing of the system is the steered MD (76,77,78). As in metadynamics, this method makes use of a time-dependent bias; in this case, an external force applied to a chosen coordinate r(x) of the system is able to move that coordinate with a constant velocity v:

$$h_t(r) = \frac{1}{2}k\left[r(x) - r(vt_t)\right]^2 \quad (6)$$

This method is appealing since it makes use of a recent formula in statistical mechanics that correlates equilibrium properties, such as the free energy difference, with a non-equilibrium procedure, such as the movement with an external bias, or the work W calculated over a forced trajectory:

$$\left\langle e^{-\beta W} \right\rangle = e^{-\beta \Delta G} \quad (7)$$

In the case of transport of a molecule through a channel, the force is applied to the molecule in the direction of the axis of diffusion of the channel. In principle, this is the best situation, given that the diffusion is along a well defined direction. While its main limitation is that it applies to a single coordinate, this technique presents some problems. In particular, when applied to a system where the biased molecule travels over a long path with different deep minima, it turns out that after traversing one transition state, the molecule does not have the time to relax before to encounter another barrier. A possible correction is to use several simulations to improve the statistics which may also change the direction of the bias, the forward-reversal method (79,80). Another problem is where to apply the bias, especially for long molecules. Schulten and coworkers applied steered MD to see how DNA translocates through alpha-emolysin, and the mechanism depends crucially on whether the bias is applied to the center of mass of DNA or to the two ends (81,82). Recently, the three methods have been compared for the first time using a test case, the transport of potassium ions in the selectivity filter of Kcsa channel (83).

6. OMPF TRANSPORT PROPERTIES BY MD SIMULATIONS

6.1. Ion transport

A series of two excellent articles published by Im and Roux touched in a nice way on the problem of transport properties through OmpF (84,85). The authors used different theoretical methods to investigate the transport of ions through OmpF. They used three approaches with decreasing details, (i) all-atom MD simulations with ions, protein and solvent explicit, (ii) Brownian dynamics with ions explicit plus a continuum for protein and solvent, and (iii) Poisson-Nerst-Planck electrodiffusion theory where all is represented as a continuum. The most demanding all-atom MD simulations have been used to evaluate some key parameters for the other simulations. The MD simulations, 5 ns for a system of approximately 70,000 atoms, at that time represented a challenge for simulations. While this simulation was static in nature, they calculated only the probability that ions occupy certain regions of OmpF, and the high number of ions used (1 M concentration) allowed improvement in the statistic.

The results they obtained with the different methods provide a consistent scenario. Simulations revealed that chloride and potassium ions move along the channel following different paths over the whole 40 Å length of the channel. Potassium ions preferably occupy the centre of the pore while chloride stay near the wall. In particular, chlorides are always coupled with a potassium ion that screens them from the high negative charge of the porin. Ion trajectories down the pore have a left-handed screw-like shape with a rotation of 180 degrees from the extracellular to the periplasmic side. At the constriction region, due to the high transversal electric field, charge separation is very crisp (see Figure 8).

6.2. Molecule transport

As described in the previous section, ion transport properties can be obtained using standard MD simulations. There, diffusion in general is controlled by many small barriers and the time-scale problem can be solved increasing ions concentration. It is the same approach as moving from single-molecule experiments to

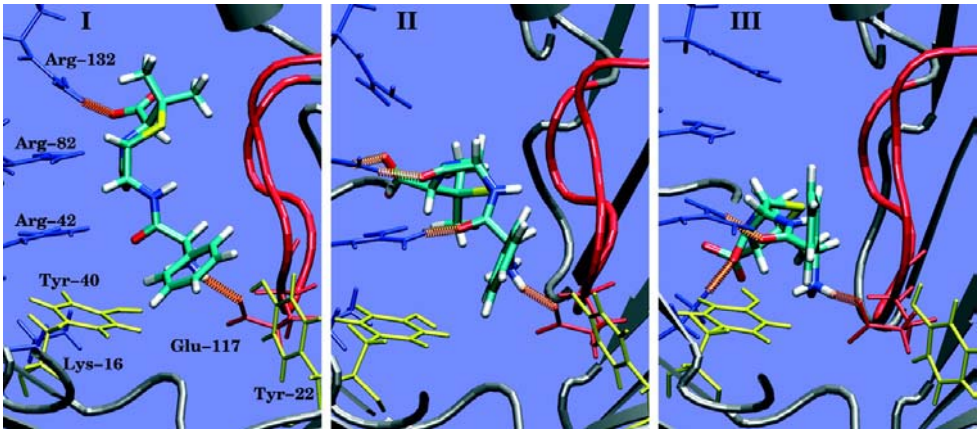


Figure 9. Characteristic conformations of Ampicillin in the constriction region during the translocation process. Confomer I is the starting conformation and shows the high matching between Ampicillin and the charged residues of the constriction region of OmpF. Reproduced with permission from Ref. 23.

multichannels, and the statistics are improved simply by increasing the number of possible events. Two problems arises when dealing with antibiotics: (i) channels have single-occupancy, and (ii) the translocation process does not seem to proceed over small barriers. This was also observed for ions by Im and Roux: when the diffusion constant of ions inside OmpF is calculated using the traditional mean square displacement, this value is 10% of the bulk value. Correcting the diffusion constant for the fact that ions move over a free energy surface with ion traps, the value becomes 50% of the bulk value: the slow diffusion of ions inside OmpF is created principally by ion trapping, or high barriers. Whether single ions can be trapped during the translocation of OmpF, with molecule is even worst and a single transport event is per se very difficult to obtain with simulations, and classified as rare events.

The first simulations of explicit molecular transport in OmpF were performed using alanine and alpha-methylglucose (86). Their dimensions are smaller than the pore size and both molecules are zwitterionic. The authors used the steered molecular dynamics simulations to investigate transport properties (76,77). From the analysis of reactive trajectories, the information obtained does not differ from previous simulations using ions (84,87). The constriction region is the bottleneck of the process with the charged groups affecting the diffusion. The molecule dipole orients in the constriction region with the transversal electric field slowing down the diffusion of the molecule. However, no binding is observed during the translocation through the pore. This can be due either to the small dimension of the molecule and/or to too fast pulling of the molecule. In this case, the authors did not apply any correction, as in the forward-reverse method (80), to take into account the relaxation problems.

6.3. Antibiotic transport

6.3.1 Static modelling

Antibiotics are small molecules with many groups whose physico/chemical properties embrace a large diversity. This is illustrated in Figure 3 for beta-lactams, but remains valid for other families of antibiotics which present charged groups and polar groups as well aromatic and hydrophobic groups. Bezrukov and co-workers (19), on the basis of their experiments, proposed a mechanism for antibiotic transport in OmpF through an internal binding-site, and the modelling of the antibiotic-OmpF system has highlighted a possible binding-site. Starting from the crystallographic structure of OmpF solved at 2.4 A, they docked ampicillin by hand near the constriction region. They positioned ampicillin transversally and with the dipole matching the charged groups of OmpF in order to follow the experimental suggestions that (i) the molecule is able to completely close the pore to the passage of ions, and (ii) there exists strong interactions to create a complex that is stable for hundreds of microseconds. Next, they minimized the whole system and refined their relative position by molecular dynamics simulations. This modelling essentially showed a high matching between ampicillin and OmpF at the constriction region, which explained the blockage time observed with experiments (see Figure 9 panel I).

6.3.2. Dynamic modelling

The above modelling lacks a dynamical description, while it is well known that proteins are dynamical entities. Using metadynamics MD simulations, the target of the investigation can be enlarged from structural properties of the constriction region (looking for a specificity of antibiotics) to dynamical properties in order to examine how antibiotics escape from that region.

Metadynamics was used to find a reactive path for the escape of ampicillin down from the constriction region of OmpF (23). The starting structure for ampicillin in the constriction region essentially assumes the previous data, see Figure 9 panel I. As stated above, metadynamics has allowed to consider more than one coordinate in a natural way. The coordinates accelerated in metadynamics reflect the initial structure; since there are many hydrogen bonds formed between ampicillin and OmpF, there is a low probability of breaking them and moving to another

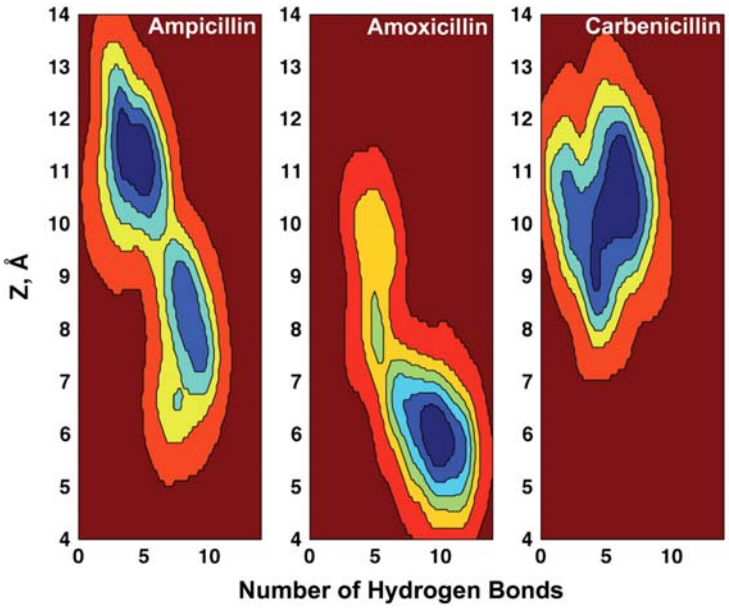


Figure 10. Contour plot of the free energy surfaces of the three antibiotics above the constriction region as obtained from metadynamics simulations. The difference between each successive color is 1 Kcal/mol. Reproduced with permission from Ref. 56.

position. This variable represents one slow mode for the translocation and hence is a natural coordinate to consider. The second coordinate is the position of the ampicillin along the z-axis of diffusion, which must change in order to observe translocation.

Accelerated MD simulations proved the passage of ampicillin through the constriction region with a simulation lasting a few nanoseconds (metatime) (see Figure 9). The main results obtained can be summarized in four points: (i) during the translocation, no large conformational change of the pore is observed. In particular, loop L3, which is responsible for the small size of the central region, does not show any large movement with respect to the whole OmpF; (ii) the barrier encountered by ampicillin in exiting from the binding-site is on the order of 10/12 Kcal/mol. Using the transition state theory, this corresponds to a typical time of hundreds of microseconds, which agrees well with experiments; (iii) the flexibility of the molecule seems to help translocation through the central region. The same simulation performed with an internal dihedral angle of ampicillin constrained to a fixed value showed a higher barrier. Due to the small size of the constriction region, the molecule is expected to have a lower entropy there, and its flexibility helps it to move from that conformation; (iv) the charged residues are important in locations other than near the constriction region, as for example the role of Lys-16 in the escape process (87) (see Figure 9, panel III).

6.4. Transport vs. molecular properties

In a more recent paper (56), modellers and experimentalists combined metadynamics and electrophysiology to compare antibiotics with different molecular properties, as seen in Figure 3. Apart from ampicillin, they used amoxicillin, which is less hydrophobic, and carbenicillin, which is anionic with a charge of -2. Experimentally, amoxicillin has a lower residence time with respect to ampicillin, at 50 microseconds compared to 120 microseconds. The case of carbenicillin is special in some way since it does not show any blockage. Metadynamics simulations were performed using the same reaction coordinates, number of hydrogen bonds per molecule/channel, and position of the centre of mass along the axe of diffusion, and with the same initial docked structures as for ampicillin. The new simulations were also extended above the constriction region, thus obtaining different free energy surfaces, see Fig 10.

For ampicillin, there is an extended binding site in the upper region, with the phenyl group that switches between Tyr22 and Tyr40 or Tyr22 and Tyr32, see Figure 11. Amoxicillin shows a binding site closer to the constriction region, favoured by the presence of the OH group that interacts more strongly with Tyr22 and Tyr40. The behaviour of carbenicillin is completely different in that once placed near the constriction region; it moves 5 Å up, due to the unfavourable interaction of its carboxylic groups with Glu117 and Asp113. Carbenicillin, moving up, finds a very strong binding site, interacting at the same time with the three arginines of the constriction region and Arg167 and Arg168 see Fig 11. Again, other amino acids not located in the constriction region seem to have a role. Moreover, carbenicillin stays near the constriction region with its longest axis parallel to the axis of diffusion Z, in contrast to the other two antibiotics that can stay perpendicular to the axis of diffusion, oriented by the electric field (see Figure 11). Apart from the lower affinity of carbenicillin for the constriction region (the bottleneck for translocation), it assumes a conformation that does not

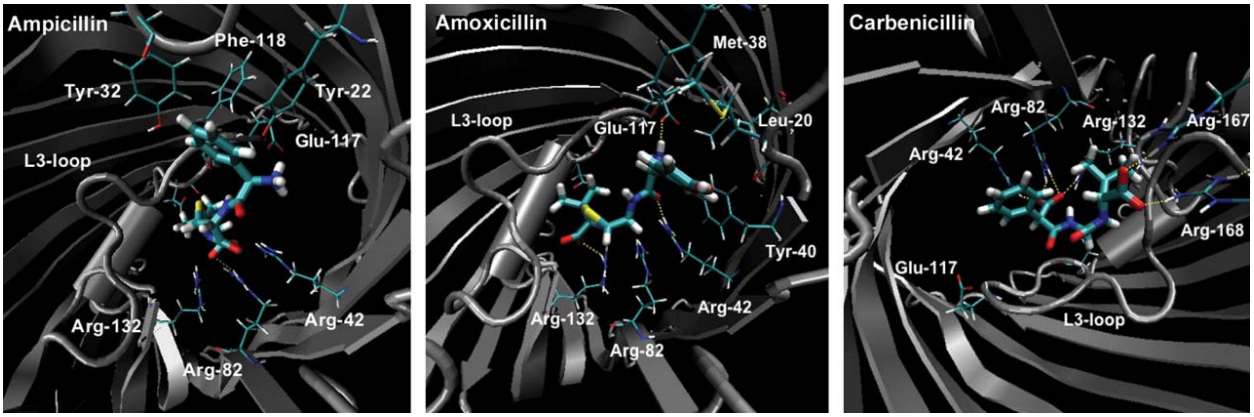


Figure 11. Top view of the most probable conformers for the three antibiotics and extracted from MD simulations. Carbon atoms are colored by cyan, hydrogens by white, nitrogens by blue, oxygen by red, and sulfur by yellow. Reproduced with permission from Ref. 56.

completely close the pore size to the passage of ions. In principle, one would have a translocation without any experimental blockage. Alternatively, following the hints of analytical models, when the binding site is not near the constriction region, the flux decreases (22). However, it is also questionable whether carbenicillin, charged at -2, is attracted by the OmpF, which is charged at -10. Carbenicillin permeation in OmpF remains an open question.

The above investigations have proved that the three antibiotics, while differing by a few external atoms, experience different interactions at the constriction regions providing different experimental blockage times, i.e. different fluxes. Therefore it would be possible to tune the flux of a molecule by changing the molecule/channel interactions. This principle can be used positively to bias drug design toward compounds with improved membrane-penetration characteristics, combining experiments with *in silico* data (7).

7. CONCLUSIONS AND PERSPECTIVES

The problem of bacterial resistance to antibiotics is an important matter, and the challenge for our society is to solve that problem by finding a new strategy to discover antibiotics. This new strategy requires more scientific knowledge of the resistance mechanisms, especially at a molecular scale. This review focuses on the techniques used and results obtained to increase our knowledge as to how antibiotics penetrate bacteria. The permeation power of antibiotics does not solely solve the bacterial resistance problem, but it represents a first step in this direction. The field of antibiotic transport through general channels is fairly new. Recent experimental techniques combined with numerical simulations seem to represent a promising and new path to increase our knowledge (19,23,56). In particular, the new algorithms that allow acceleration of MD simulations (63) can be very useful for gaining details at the molecular scale in the translocation process of antibiotics. As shown, the agreement with experiments is quite good, and simulations might have a high potentiality in the future design of new antibiotics with improved transport properties.

The multidisciplinary approach that combines experiments, MD simulations, and analytical models, is extremely attractive and powerful in offering many details of the molecular processes. However, a gap with *in vivo* experiments remains. Many issues are open, such as the role of the asymmetric lipopolysacharides membrane of bacteria in the transport properties (16,89,90). To date, electrophysiology experiments have been performed by reconstructing OmpF in a symmetric bilayer (19,56). Second, OmpF is not the only general channel used by antibiotics to penetrate bacteria. OmpC, for which an X-ray structure recently appeared, is another potential path for antibiotics uptake (91). Also, OmpT and OmpU modelled by homology, are also potentially good targets (92,93), as well OmpW and OmpX (94). Moving to antibiotics, there are more families to be considered, such as fluoroquinolones and the last generation of cephalosporines (30,36).

8. ACKNOWLEDGMENTS

This work was supported by project grants from European Community (Research Training Network 019335 “Translocation” 2006-2009) and partially from Italian MUR through the project PON-CYBERSAR. Simulations were performed at the CASPUR computer center, Roma (Italy), and COSMOLAB computer center, Cagliari (Italy).

9. REFERENCES

1. Page, M.: Superbugs: apocalipse now or the day after tomorrow? *Europ. BioPharm. Rev. Spring Issue*, 40-45 (2005)

2. See www.idsociety.org/badbugsnodrugs

3. Brad Spellberg B., J. H. Powers, Eric P. Brass, Loren G. Miller & J. E. Edwards Jr: Trends in Antimicrobial Drug

Development: Implications for the Future. *Clin. Infect. Dis.* 38, 1279-1286 (2004)

4. D'Costa V., K. M. McGrann, D. W. Hughes & G. D. Wright: Sampling the antibiotic resistore. *Science* 311, 374-377 (2006)

5. Vicente M., J. Hodgson, O. Massidda, T. Tonjum, B. Henriques-Normark & E. Z. Ron: The fallacies of hope: will we discover new antibiotic to combat pathogenic bacteria in time? *FEMS Microbiol. Rev.* 30, 841-852 (2006)

6. Payne, D. J., M. N. Gwynn, D. J. Holmes & D. L. Pompeiano: Drugs for bad bugs: confronting the challenges of antibacterial discovery. *Nature Rev. Drug Discovery* 6, 29-40 (2007)

7. Barker, J. J.: Antibacterial drug discovery and structure-based design. *Drug. Discov. Today* 11, 391-404 (2006)

8. Davies J.: Inactivation of antibiotics and the dissemination of resistance genes. *Science,* 264, 375-382 (1994)

9. Hermann J. C., C. Hensen, L. Ridder, A. J. Mulholland & H.-D Höltje: Mechanisms of antibiotic resistance: qm/mm modeling of the acytalion reaction af a class of A beta-lactamase with benzylpenicillin. *J. Am. Chem. Soc,* 127, 4454-4465 (2005)

10. Nikaido, H.: Outer membrane barrier as a mechanism of antimicrobial resistance. *Antimicrob. Agents Chemoter.* 33, 1831-1836 (1989)

11. Webber M. A. & L. J. Piddock: The importance of efflux pumps in bacterial antibiotic resistance. *J Antimicrob Chemother.* 51, 9-11 (2003)

12. Delcour, A. H.: Solute uptake through general porins. *Front. Biosi.* 8, 1055-1071 (2003)

13. Koronakis, V., A. Sharff, E. Koronakis, B. Luisi & C. Hughes. 2000: Crystal structure of the bacterial membrane protein TolC central to multidrug efflux and protein export. *Nature* 405, 914–919 (2000)

14. Murakami, S., R. Nakashima, E. Yamashita & A. Yamaguchi: Crystal structure of bacterial multidrug efflux transporter AcrB. *Nature* 419, 587–593 (2002)

15. Pos, K. M., A. Schiefner, M. A. Seeger & K. Diedrichs: Crystallographic analysis of AcrB. *FEBS Lett.* 564, 333–339 (2004)

16. Nikaido, H.: Prevention of drug access to bacterial targets: permeability barriers and active efflux. *Science* 264, 382-388 (1994)

17. Simonet V., M. Malléa & J.-M. Pagès: Substitutions in the eyelet region disrupt cefepime diffusion through the *Escherichia Coli* OmpF channel. *Antimicrob. Agents Chemoter.* 44, 311-315 (2000)

18. Piddock L. J.: Clinically relevant chromosomally encoded multidrug resistance efflux pumps in bacteria. *Clin. Microbiol. Rev.* 19, 382-402 (2006)

19. Nestorovich, E. M., C. Danelon, M. Winterhalter & S. M. Bezrukov: Designed to penetrate: time-resolved interaction of single antibiotic molecules with bacterial pores. *Proc. Natl. Acad. Sci. USA*. 99, 9789-9794 (2002)

20. Schwarz, G. C., C. Danelon & M. Winterhalter: On translocation through a membrane channel via an internal binding site: kinetics and voltage dependence. *Biophys. J.* 84, 2990-2998 (2003)

21. Berezhkovskii, A. M. & S. M. Bezrukov: Optimizing Transport of metabolites through Large Channels: Molecular Sieves with and without Binding. *Biophys. J.* 104, L17-L19 (2005)

22. Bauer, W. R. & W. Nadler: Molecular transport through channels and pores: effects of in-channel interactions and blocking. *Proc. Natl. Acad. Sci. USA* 103, 11446-11451 (2006)

23. Ceccarelli, M., C. Danelon, A. Laio & M. Parrinello: Microscopic mechanism of antibiotics translocation through a porin. *Biophys. J.* 87, 58–64 (2004)

24. Berg J. M., J. L. Tymoczko & L. Strayer: Biochemistry. W. H. Freeman and Company New York (2002)

25. Luckey M. & H. Nikaido: Specificity of diffusion channels produced by X phage receptor protein of Escherichia coli. *Proc. Natl. Acad. Sci. USA* 77, 165-171 (1980)

26. Benz R., A. Schmid & G. H. Vos-Sheperkeuter: Mechanism of sugar transport through the sugar-specific LamB channel of Escherichia coli outer membrane. *J. Membr. Biol.* 100, 21-29 (1987)

27. Dutzler R., Y. F. Wang, P. J. Rizkallah, J. P. Rosenbusch & T. Schirmer: Crystal structures of various maltooligosaccharides bound to maltoporin reveal a specific sugar translocation pathway. *Structure* 4, 127-134 (1996)

28. Dutzler R., T. Schirmer, M. Karplus & S. Fischer: Translocation mechanism of long sugar chains across the maltoporin membrane channel. *Structure* 10, 1273-1284 (2002)

29. Danelon C., T. Brando & M. Winterhalter: probing the orientation of reconstituted maltoporin channels at the single-protein level. *J. Biol. Chem.* 278, 35542-35551 (2003)

30. Jacoby G. A.: Mechanism of resistance to quinolones. *Clin. Infect. Dis.* 41, S120-S126 (2005)

31. Cowan S. W., T. Schirmer, G. Rummel, M. Steiert, R.A. Pauptit, J. N. Jansonius & J. P. Rosenbusch: Crystal

structures explain functional properties of two *E. coli* porins. *Nature* 358, 727-733 (1992)

32. Watanabe M., J. Rosenbusch, T. Schirmer & M. Karplus: Computer simulations of the ompf porin from the outer membrane of *Escherichia coli. Biophys. J.* 72, 2094-2102 (1997)

33. Tieleman D. P. & H. C. J. Berendsen: A molecular dynamics study of the pores formed by *Escherichia coli* ompf porin in a fully hydrated palmitoyloleoylphosphatidylcholine bilayer. *Biophys. J.* 74, 2786-2801 (1998)

34. Nikaido H.: Molecular basis of bacterial outer membrane permeability revisited. *Microbiol. Mol. Biol. Rev.* 67, 593-656 (2003)

35. Karshikoff A., V. Spassov, S. W. Cowan, R. Ladenstein & T. Schirmer: Electrostatic properties of two porin channels from *Escherichia coli. J. Mol. Biol.* 240, 372-384 (1994)

36. Vidal S., J. Bredin, J.-M. Pagès & J. Barbe: beta-lactam screening by specific residues of the OmpF eyelet. *J. Med. Chem*., 48, 1395-1400 (2005)

37. Hanke W. & W. R. Schlue: Planar lipid bilayers: methods and applications. Academic Press, San Diego, CA (1993)

38. Benz R., K. Janko, W. Boos & P. Lager: Formation of large, ion-permeable membrane channels by the matrix protein (porin) of *Escherichia coli. Biochim. Biophys. Acta* 511, 305-319 (1978)

39. Schindler H. & J. P. Rosenbusch: Matrix protein in planar membranes: clusters of channels in native environment and their functional reassembly. *Proc. Natl. Acad. Sci. USA*. 78, 2302-2306 (1981)

40. Nikaido H. & E. Y. Rosenberg: Porin channels in *Escherichia coli:* studies with liposomes reconstituted from purified proteins. *J. Bacteriol.* 153, 241-252 (1983)

41. Yoshimura F. & H. Nikaido: Diffusion of beta-lactam antibiotico through the porin channels of *Escherichia coli*. *Antimicrob. Agents Chemother.* 27, 84-92 (1985)

42. Winterhalter M.: Sugar transport through channels reconstituted in planar lipid membranes. *Colloide and Surfaces A* 149, 547-551 (1999)

43. Bezrukov S. M.: Ion channels as molcular Coulter counters to probe metabolite transport. *J. Membrane Biol.* 174, 1-13 (2000)

44. Bezrukov S. M. & M. Winterhalter: Examining noise sources at the single-molecule level: 1/f noise of an open maltoporin channel. *Phys. Rev. Lett.* 85, 202-205 (2000)

45. Hilty C. & M. Winterhalter: Facilitated substrate transport through membrane proteins. *Phys. Rev. Lett.* 86, 5624-5627 (2001)

46. Benz R., A. Schmid, T. Nakae & G. H. Vos-Scheperkeuter: Pore formation by LamB of *Escherichia coli* in lipid bilayer membranes. *J. Bacteriol.* 165, 978-986 (1986)

47. Andersen C., M. Jordy & R. Benz: Evaluation of the Rate Constants of Sugar Transport Through Maltoporin (LamB) of *Escherichia coli* from the Sugar-induced Current Noise. *J. Gen. Physiol.* 105, 385-401 (1995)

48. Kullman L., M. Winterhalter & S. M. Bezrukov: Transport of maltodextrins through maltoporin: a single-channel study. *Biophys. J.* 82, 803-812 (2002)

49. Bolhuis, P. G., C. Dellago, & D. Chandler: Sampling ensembles of deterministic transition pathways. *Faraday Discuss*. *110*, 421-436 (1998)

50. Dellago, C., P. G. Bolhuis, F. S. Csajka & D. Chandler: Transition path sampling and the calculation of rate constants. *J. Chem. Phys. 108*, 1964-1977 (1998)

51. Zahn D.: How Does Water Boil? *Phys. Rev. Lett. 93*, 227801 (2004)

52. Zahn D.: Competing Evaporation and Condensation Processes during the Boiling of Methane. *J. Phys. Chem. B 110,* 19601-19604 (2006)

53. Berezhkovskii A. M., G. Hummer & S. M. Bezrukov: Identity of distributions of direct uphill and downhill translocation times for particles traversing membrane channels. *Phys. Rev. Lett.* 97, 020601 (2006)

54. Kosztin, I. & K. Schulten: Fluctuation-driven lolecular transport through an asymmetric membrane channel. *Phys. Rev. Lett*. 93:238102 (2004)

55. Kolomeisky A. B.: Channel-facilitated molecular transport across membranes: attraction, repulsion, and asymmetry. *Phys. Rev. Lett*. 98:048105 (2007)

56. Danelon, C., E. M. Nestorovich, M. Winterhalter, M. Ceccarelli & S. M. Bezrukov: Interaction of zwitterionic penicillins with the OmpF channel facilitates their translocation. *Biophys. J.* 90, 1617-1627 (2006)

57. Allen M. P. & D. J. Tildesley: Computer simulation of liquids. *Oxford Science Publication* (1986)

58. Karplus M. & G. A. Petsko: Molecular dynamics simulations in biology. *Nature* 347, 631-639 (1990)

59. Chandler D.: Introduction to modern statistical mechanics. *Oxford University Press* (1987)

60. Marrink S.-J. & H. J. C. Berendsen: Simulation of water transport through a lipid membrane. J. Phys. Chem. 98, 4155-4168 (1994)

61. Hanggi P., P. Talkner & M. Borkovec: Reaction-rate theory: fifty years after Kramers. *Rev. Mod. Phys.* 62, 251-341 (1990)

62. Chandler D.: Computer simulations of rare events and the dynamics of classical and quantum condensed matter systems, Lecture 3. Proceedings of the Enrico Fermi Summer School (1997)

63. Laio A. & M. Parrinello: Escaping free-energy minima. *Proc. Natl. Acad. Sci. USA* 99, 12562-12566 (2002)

64. Laio, A., A. Rodriguez-Fortea, F. L. Gervasio, M. Ceccarelli & M. Parrinello: Assessing the accuracy of metadynamics. *J. Phys. Chem. B* 109, 6714-6721 (2005)

65. Gervasio, F. L., A. Laio & M. Parrinello: Flexible Docking in Solution Using Metadynamics. *J. Am. Chem. Soc. 127*, 2600-2607 (2005)

66. Branduardi, D., F. L. Gervasio, A. Cavalli, M. Recanatini & M. Parrinello: The role of the peripheral anionic site and cation-pi interactions in the ligand penetration of the human AChE Gorge. *J. Am. Chem. Soc.* 127, 9147-9155 (2005)

67. Barducci, A., R. Chelli, P. Procacci, V. Schettino, F. L. Gervasio & M. Parrinello: Metadynamics simulation of prion protein: beta-structure stability and the early stages of misfolding. *J. Am. Chem. Soc.* 128, 2705-2710 (2006)

68. Ceccarelli M., R. Anedda, M. Casu & P. Ruggerone: CO escape from myoglobin with metadynamics simulations. *Prot. Struct. Funct. Bioinf.* 71, 1231-1236 (2008)

69. Torrie, G. M. & J. P. Valleau: Nonphysical sampling distributions in Monte Carlo free-energy estimation: Umbrella sampling. *J. Comp. Phys. 23*, 187-199 (1977)

70. Kumar, S., D. Bouzida, R. H. Swensen, P. A. Kollman & J. M. Rosenberg: The weighted histogram analysis method for free-energy calculations on biomolecules. I. The method. *J. Comp. Chem. 13*, 1011-1021 (1992)

71. Souaille, M. & B. Roux: Extension to the weighted histogram analysis method: combining umbrella sampling with free energy calculations. *Comput. Phys. Commun. 135*, 40-57 (2001)

72. Raiteri, P., A. Laio, F. L. Gervasio, C. Micheletti & M. Parrinello: Efficient Reconstruction of Complex Free Energy Landscapes by Multiple Walkers Metadynamics. *J. Phys. Chem. B* 110, 3533-3539 (2006)

73. Piana S. & A. Laio: A Bias-Exchange Approach to Protein Folding. *J. Phys. Chem. B* 111, 4553-4559 (2007)

74. Bussi, G., F. L. Gervasio, A. Laio & M. Parrinello: Free-energy landscape for beta-hairpin folding from combined parallel tempering and metadynamics. *J. Am. Chem. Soc. 128*, 13435-13441 (2006)

75. Babin, V., C. Roland, T. A. Darden & C. Sagui: The free energy landscape of small peptides as obtained from metadynamics with umbrella sampling corrections. *J. Chem. Phys. 125*, 204909 (2006)

76. Jarzynski. C.: Nonequilibrium equality for free energy differences. *Phys. Rev. Lett.* 14, 2690-2693 (1997)

77. Hummer, G. & A. Szabo: Free energy reconstruction from nonequilibrium single-molecule experiments. *Proc. Natl. Acad. Sci. USA* 98, 3658-3661 (2001)

78. Park S. & K. Schulten: Calculating potentials of mean force from steered molecular dynamics simulations. *J. Chem. Phys* 120, 5946- 5948(2004)

79. Crooks, G.E.: Nonequilibrium Measurements of Free Energy Differences for Microscopically Reversible Markovian Systems. *J. Stat. Phys.* 90, 1481-1487 (1998)

80. Kostzin, I., B. Barz & L. Janosi: Calculating potentials of mean force and diffusion coefficients from nonequilibrium processes without Jarzynski's equality. *J. Chem. Phys.* 124, 064106 (2006)

81. Mathe J., A. Aksimentiev, D. R. Nelson, K. Schulten & A. Meller: Orientation discrimination of single stranded DNA inside the alpha-hemolysin membrane channel. *Proc. Natl. Acad. Sci. USA* 102, 12377-12382 (2005)

82. Heng. J. B., A. Aksimentiev, C. Ho, P. Marks, Y. V. Grinkova, S. Sligar K. Schulten & G. Timp: Stretching DNA using an electric field in a syntethic nanopore, *Nano Letters* 5, 1883-1888 (2005)

83. Piccinini, E., M. Ceccarelli, F. Affinito, R. Brunetti & C. Jacoboni: Biased Molecular Simulations for Free-Energy Mapping: A comparison on the KcsA channel as a test Case. *J. Chem. Theory Comput*., accepted (2007)

84. Im W. & B. Roux: Ions and counterions in a biological channel: a molecular dynamics simulation of OmpF porin from Escherichia coli in an explicit membrane with 1 M KCl aqueous salt solution. *J. Mol. Biol.* 319, 1177-1197 (2002)

85. Im W. & B. Roux: Ion permeation and selectivity of OmpF porin: a theoretical study based on molecular dynamics, Brownian dynamics, and continuum electrodiffusion theory. *J. Mol. Biol.* 322, 851-869 (2002)

86. Robertson K. M. & D. P. Tieleman: Orientation and interactions of dipolar molecules during transport through OmpF porin. *FEBS Lett.* 528, 53-57 (2002)

87. Phale P. S., A. Philippsen, C. Widmer, V. P. Phale, J. P. Rosenbusch & T. Schirmer: Role of charged residues at the

OmpF porin channel constriction probed by mutagenesis and simulation. *Biochemistry* 40, 6319-6325 (2001)

88. Bredin J., N. Saint, M. Malléa, E. Dé, G. Molle, J.-M. Pagès & V. Simonet: Alteration of pore properties of *Escherichia coli* OmpF induced by mutation of key residues in anti-loop 3 region. *Biochem. J.* 363, 521-528 (2002)

89. Tong J. & T. J. McIntosh: Structure of supported bilayers composed of lipopolysaccharides and bacterial phospholipids: raft formation and implications for bacterial resistance. *Biophys. J.* 86, 3759-3771 (2004)

90. Lins R. D. & T. P. Straatsma: Computer Simulation of the Rough Lipopolysaccharide Membrane of *Pseudomonas aeruginosa. Biophys. J.* 81, 1037–1046 (2001)

91. Baslé A., G. Rummel, P. Storici, J. P. Rosenbusch & Tilman Schirmer: Crystal structure of osmoporin OmpC from E. coli at 2.0 Å. *J. Mol. Biol.* 362, 933-942 (2006)

92. Duret, G. & A. H. Delcour: Deoxycholic acid blocks vibrio cholerae OmpT but not OmpU porin, *J. Biol. Chem.,* 281, 19899-19905 (2006)

93. Simonet, V. C., A. Baslé, K. E. Klose & A. H. Delcour: The vibrio cholerae porins OmpU and OmpT Have distinct channel properties. *J. Biol. Chem*. 278, 17539-17545 (2003)

94. Hu W. S., P.-C. Li & C.-Y. Cheng: Correlation between ceftriaxone resistance of *Salmonella enterica* serovar typhimurium and expression of outer Membrane proteins OmpW and Ail/OmpX-like protein, which are regulated by BaeR of a two-Component System. *Antimicrob. Agents Chemoter*. 49, 3955–3958 (2005)

Key Words: Antibiotics, Bacteria, Porins, Single-Molecule Electrofisiology, Transport Properties, Molecular Dynamics, Free-Energy, Review

Send correspondence to: Matteo Ceccarelli, Dipartimento di Fisica and SLACS-INFM, Universita degli Studi di Cagliari, Cittadella Universitaria di Monserrato, SP Monserrato-Sestu Km 0.700, 09042 Monserrato, Italy, Tel: 390706754933, Fax: 39070510171, E-mail: matteo.ceccarelli@dsf.unica.it