Eur Biophys J (2009) 38:1141–1145
DOI 10.1007/s00249-009-0495-0

BIOPHYSICS LETTER

Antibiotic translocation through membrane channels: temperature-dependent ion current fluctuation for catching the fast events

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Received: 16 April 2009 / Revised: 16 May 2009 / Accepted: 18 May 2009 / Published online: 9 June 2009

Abstract Temperature-dependent facilitated permeation of antibiotics through membrane channels was investigated. Here we reconstituted single OmpF trimers from the outer membrane of *Escherichia coli* (*E. coli*) into a planar lipid bilayer. The penetration of ampicillin through OmpF causes fluctuation in the ion current, and analysis of the fluctuations at different temperatures allows us to determine the mode of permeation. The residence time of the drug inside the channel decays strongly with temperature, reaching the resolution limit of the instrument at 30°C. The number of events increases exponentially with temperature up to 30°C and then gradually decreases as temperature increases. At room temperature, we observe about 25 events per second per monomer of the trimeric channel and an extrapolation to 37°C gives roughly 50 events. The activation energy for ampicillin translocation through OmpF is estimated to be around 13 *kT*. Temperature-dependent study gives new insights into the faster translocation of small substrates through biological nanopores.

Keywords Arrhenius · Temperature · OmpF-ampicillin translocation (nonspecific)

Electronic supplementary material The online version of this article (doi:10.1007/s00249-009-0495-0) contains supplementary material, which is available to authorized users.

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Introduction

Selectivity is a key parameter in biology. A particular example is transport across the outer cell wall of bacteria with the need to discriminate between the uptake of nutrition and toxic substances (Saier 2007). In Gram-negative bacteria, a first molecular sieve is their outer membrane, consisting of water-filled channels embedded in an asymmetric lipid bilayer (Yoshimura and Nikaido 1985; Nikaido 2003). This membrane acts as an impermeable barrier for water-soluble molecules, while channels called porins allow facilitated diffusion of nutrition into the periplasm (Yoshimura and Nikaido 1985; Nikaido 2003). The success of an antibiotic depends among other factors on how it overcomes the protective outer membrane barrier. Minimal inhibitory concentration (MIC) studies on porin-deficient mutants with antibiotics provide strong evidence for the role of porins in antibiotic translocation (Pagès et al. 2008; James et al. 2009). More specifically it has been shown that the general diffusion porin OmpF serves as a major gateway for the passage of beta-lactam antibiotics and fluoroquinolones in *E. coli* (Yoshimura and Nikaido 1985; Nikaido 2003; Pagès et al. 2008). The three-dimensional structure of the OmpF has been determined and the conserved L3 loop constitutes a crucial part in the influx of antibiotics, as shown by sequence analysis of resistant strains (Cowan et al. 1992; Bredin et al. 2002; Phale et al. 2001). Earlier studies with reconstituted proteoliposomes (liposome swelling assays) indicated that zwitterionic compounds penetrate very rapidly compared with other charged compounds (Yoshimura and Nikaido 1985). All these methods revealed qualitative values showing the relevance of the channel or mutations to the antibiotic activity.

An appropriate technique to characterize channel-forming proteins is electrophysiology; for example, channels may be reconstituted into an artificial planar lipid bilayer and the ion current through the channel can be measured (Rostovtseva et al. 2002; Nestorovich et al. 2003; Danelon et al. 2003a). Addition of sugars or other molecules supposed to permeate through the channel will cause concentration-dependent complete or partial blocking of the ion current (Nekolla et al. 1994; Danelon et al. 2003b; Nestorovich et al. 2002). Using the ion current fluctuation as a molecular sensor of channel occupation allows quantitative conclusions to be made on the mode of permeation. This method has been used to quantify maltooligosacharose permeation through maltoporin, adenosine triphosphate (ATP) through mitochondrial porins, and more recently a number of antibiotics through OmpF (Nestorovich et al. 2002; Danelon et al. 2006; Mach et al. 2008).

Translocation of several beta-lactam antibiotics and the fluoroquinolone moxifloxacin through OmpF was studied using ion current noise analysis (Nestorovich et al. 2002; Danelon et al. 2006; Mach et al. 2008). The interaction between antibiotics and the OmpF channel was strong enough to see it as time-resolved ion current blockages. A first analysis revealed a strong correlation between the ability to block the ion current and the antimicrobial activity. Most of the investigated antibiotics causing channel blockage correlated with biological efficiency. However, in some cases more biologically active molecules revealed little or virtually no effect on ion conductance, for instance, the antibiotic penicillin G had no discernible effect.

Here we followed the hypothesis that ampicillin, an example of a water-soluble antibiotic, uses molecular interaction with the channel surface for facilitated entry into the periplasmic space. However, analyzing the rates obtained resulted in fairly low numbers at room temperature. Kinetic measurements at a relevant higher temperature revealed an even lower number of events. Here we show that this is due to the finite time resolution of this particular technique. We arrive at a corrected higher number and conversely we may slow down the kinetics by lowering the temperature, thereby opening the way to observe ion current blockages by antibiotics previously giving an inconclusive negative result.

Materials and methods

Chemicals

The following chemical reagents were used in this study: KCl, 2-(*N*-morpholino) ethanesulfonic acid (MES), potassium phosphate, *n*-pentane, hexadecane, and ampicillin anhydrous (Sigma Aldrich, Buchs, Switzerland); ampicillin sodium salt (Applichem, Darmstadt, Germany); and 1,2-diphytanoyl-sn-glycero-3-phosphatidylcholine (DPhPC) (Avanti Polar Lipids, Alabaster, AL). Doubly distilled and deionized water was used to prepare solutions. After preparation, all solutions were purified by filtration through a 0.4-mm filter. For our studies, we selected the antibiotic ampicillin, a zwitterionic compound found to be stable at measured temperatures verified by ultraviolet (UV) spectrometry.

Reconstitution experiments and noise analysis

High-resolution ion current recordings were carried out with planar lipid bilayers formed according to the technique developed by Montal and Mueller (1972) with slight modifications (Chimerel et al. 2008; Jung et al. 2006; Kang et al. 2005). The *cis* and *trans* compartments of a Teflon chamber were separated by a 25-µm-thick Teflon film (Good fellow, Cambridge, UK) containing a round aperture of 40–50 µm diameter. Most of the experiments were carried out with Teflon septum of 40 µm aperture that yielded stable bilayers with low noise prepainted with 2% squalene. The electrolyte used was 1 M KCl, 20 mM phosphate, pH 6 with solution volume of 1.5 ml. The porins were added to the *cis* chamber, to give a final concentration of 1–2 ng/ml. Porin insertion was facilitated by mixing the contents of the chamber and applying transmembrane voltage of 150–200 mV. In order to vary the temperature over a broad range we included a Peltier element for temperature regulation (Dagan) (Chimerel et al. 2008). Insertion was best at $T = 25°C$, and after the first insertion the cuvette was carefully rinsed with buffer to remove remaining porins. Subsequently the temperature was lowered to 5°C and the measurement started. The temperature was increased manually and adjusted to the new temperature. Single-channel measurements were recorded by using an Axopatch 200B amplifier (Axon Instruments, Foster City, CA) connected to Ag/AgCl electrodes (World Precision Instruments, Sarasota, FL) used to detect ion currents. The signal was filtered using a four-pole low-pass Bessel filter at a frequency of 10 kHz and sampled at 50 kHz, acquired using a Digidata 1322A digitizer, and analyzed using pClamp 10.0 software (Axon Instruments, Foster City, CA). Ion current blockage events were measured following the addition of antibiotics to the *cis* or *trans* or both sides of the lipid membrane. Ampicillin sodium salt and ampicillin anhydrous (as a control to enable comparison to (Nestorovich et al. 2002), data not shown) was used for the measurement.

Results and discussion

To gain better understanding of the antibiotic translocation, we quantified antibiotic interaction with OmpF channel at different temperatures. As described in "Materials and methods," we reconstituted single OmpF trimer into a planar lipid membrane. Before addition of ampicillin, various transmembrane voltages were applied and the corresponding ion current traces were recorded. The obtained baseline agreed with previous recordings (Chimerel et al. 2008). Fluctuations in the ion conductance through the channels were analyzed after adding antibiotic to the *cis* or *trans* side of the lipid membrane measured. As shown in Fig. 1, ampicillin addition (*cis* side) caused reversible blocking of the ion current of one monomer of a single trimeric OmpF channel at applied transmembrane voltage of +50 mV. At 5°C, relatively few ion current blockages (events), with residence times above 1 ms, are visible. Increasing the temperature to 25°C increases blockage numbers and decreases residence time of the drug in the channel. This trend is continued up to 30°C. At higher temperatures, the blocking events are shorter and less frequent. Figure 2a shows the number of events increasing with temperature but decreasing above $T = 30°C$. In contrast, in Fig. 2b the residence time decreases with the increase in temperature, which indicates that at higher temperature the time spent by the ampicillin in the channel is shorter. At the higher temperature of 55°C frequent partial monomer closure of a single trimer is observed. It is interesting to note that, within the investigated temperature range, the residence time was independent of the side of drug addition and of concentration. This indicates that OmpF possesses a single affinity site for the antibiotic molecule.

As a first approach, we may hypothesize an Arrhenius behavior for the number of events and residence times, as these are connected to the on- and off-rates of ampicillin penetration into OmpF channels. From the number of events υ we may deduce that the on-rate at a given concentration $[c]$ of ampicillin is $k_{\text{on}} = \upsilon/3[c]$ and that the off-rate $k_{\text{off}} = 1/\tau$ is the inverse of the residence time τ.

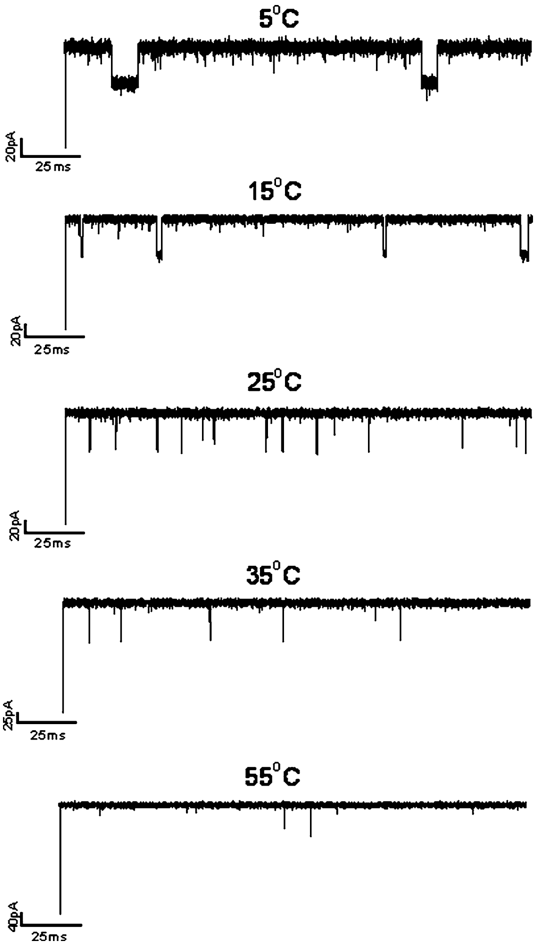

Fig. 1 Ion current fluctuation through OmpF in presence of 10 mM ampicillin. From top: 5°C, several blockage events of a few milliseconds duration occur; 15°C, more events occur but with substantially shorter residence times compared with at 5°C; 25°C, more events occur but with substantially shorter residence times below the millisecond scale; 35°C, apparent drop in the number of blocking events with even shorter residence times. This trend continues at 55°C, with fewer events and lower amplitude

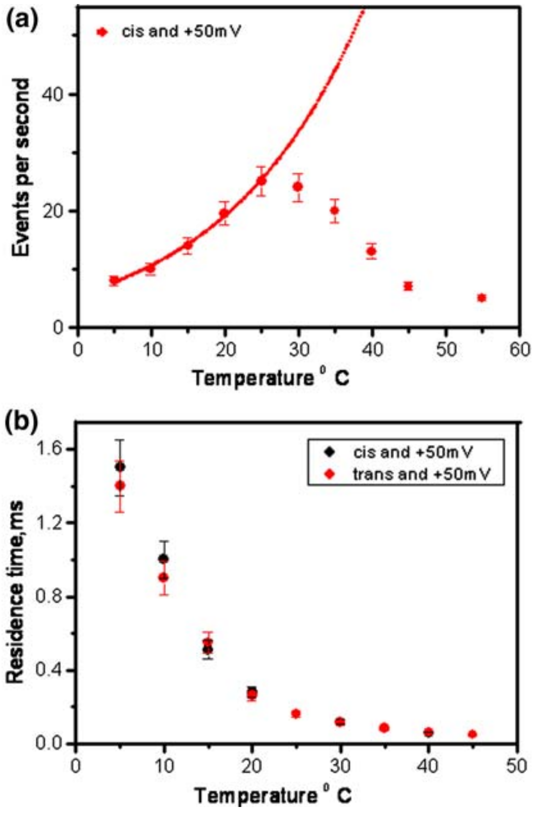

Fig. 2 Statistical analysis of the ion current fluctuations as a function of temperature at 50 mV applied potential. **a** Increasing temperature increases the number of events with a maximum at 25°C; further increase apparently reduces the number of events. The continuous line represents extrapolation of the number of events to higher temperature. **b** The average residence time was independent of the polarity of the voltage and side of antibiotic addition. Increasing temperature correlates with shorter residence time

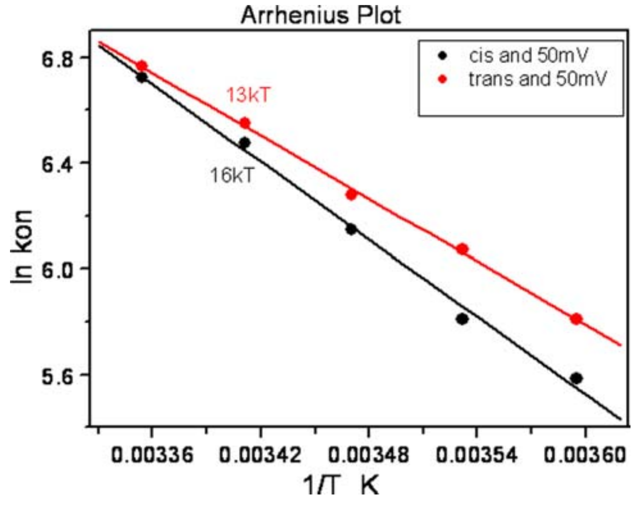

Fig. 3 Arrhenius plot of k_{on} as a function of temperature. A reduced data set shown in Fig. 2a up to 25°C resulted in a linear slope with an energy barrier of about 16 kT for *cis*-side and 13 kT for *trans*-side addition. (50 mV applied voltage)

An expected exponential increase in the event number was fitted to the low-temperature region of Fig. 2a. The strong temperature dependence of k_{on} has been correlated to an activation barrier—the Arrhenius plot corresponding to the fitted region of Fig. 2a (5–25°C) can be seen in Fig. 3. The effective energy barrier for the ampicillin translocation through the OmpF channel was calculated from this Arrhenius plot. The natural logarithm of k_{on} shows a linear dependence in $1/T$, giving E_{a} (the calculated slope times the ideal gas constant), from which we determined E_{a}^{cis} and E_{a}^{trans}, respectively (Fig. 3). This corresponds to the effective energy barrier required for the ampicillin to permeate from the *cis* (E_{a}^{cis}) or *trans* side (E_{a}^{trans}) to the affinity site in the channel. This linear fit in the 5–25°C temperature range yields an energy barrier of about 13 kT for *trans*-side addition and about 16 kT for *cis*-side addition (50 mV transmembrane voltage). The internal affinity site is accessible from the *cis* and *trans* side of the lipid membrane, and each of these access routes have different activation barriers. The kinetic difference reflected by the *cis* and *trans* on-rates demonstrates a structural asymmetry of the channel. This was pointed out in the similar case of sugar transport through maltoporin (Schwarz et al. 2003; Berezhkovskii and Bezrukov 2005; Bauer and Nadler 2006). Inspection of the experimental values for the on- and off-rates in the biological relevant concentration gradient range (μM) yields that $k_{\text{off}} >> k_{\text{on}}\ \Delta c$. This simplifies the flux to $J = k_{\text{on}}\ \Delta c/2$ (Weingart et al. 2008; Schwarz et al. 2003). In other words, increasing the on-rate will directly result in an increase in translocation and thus the drug efficiency should increase.

Drawing conclusions from the fitted data at 5–25°C reveals that the number of translocated molecules depends exponentially on temperature, increasing the number of ampicillin molecules to about 50 per second per monomer at 37°C. It is interesting to note that such temperature dependence is also observed for OmpF mutants (supplementary material). Lowering the temperature slows down the kinetics and we could show antibiotic translocation through OmpF mutants that are not visible at room temperature (supplementary figure).

The apparent decrease in event number above 30°C (Fig. 2a) contradicts the Arrhenius trend. We suggest that at high temperature the translocation is too fast to be resolved. At 30°C, the residence time drops appreciably below 100 μs (Fig. 2b), corresponding to our inverse filter frequency, and the apparent partial closures at 55°C can correspond to filter distortion and cropping of full depth closures (Hawkes et al. 1990; Colquhoun and Sigworth 1983). The almost negligible decrease in measured residence time for higher temperatures also supports this hypothesis.

While the temperature-dependent ion conductance of OmpF has been measured for a wider temperature range (Chimerel et al. 2008) we cannot at this stage rule out a local conformational change in the OmpF porin affecting only the binding site. A diffusion model could also be related to our observations (Berezhkovskii and Bezrukov 2005; Berezhkovskii et al. 2002a, 2002b). However, given the good correspondence of the kinetic rates to the Arrhenius equation and the conspicuous change in trend at the inverse filter frequency, we find an instrumental limitation more likely.

Using current blockages gives a single-molecule quantitative handle on translocation; however, the resolution limit of this technique limits the detection of events occurring below the microsecond time scale. By considering the temperature dependence of the rates, we may successfully extrapolate to higher (biologically relevant) temperatures. Conversely, we may slow down kinetics of faster translocating antibiotics by lowering the temperature. Considering that fast translocation of antibiotics without ion current blockages at room temperature is possible, temperature-dependent measurements can be successfully employed to study this faster translocation. It is interesting to note that our finding is related to current investigation on fast translocation of DNA through solid-state nanopores (Comer et al. 2009; Wanunu et al. 2008; Heng et al. 2006).

Molecular dynamics (MD) simulations have been used to study the transport properties of ampicillin and moxifloxacin through the OmpF and correlated with the kinetic rates in lipid bilayer measurements (Mach et al. 2008; Ceccarelli et al. 2004). Applying our temperature scan allows the direct calculation of activation energy barriers of a relevant translocation at a single-molecule scale. This result may be used as an input for computer modeling, thereby further increasing the synergy between these methods.

Acknowledgments This study was supported by EU-grant MRTN-CT-2005–019335 (Translocation).

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