

# Voltage-Induced “Gating” of Bacterial Porin as Reversible Protein Denaturation

Ekaterina M. Nestorovich and Sergey M. Bezrukov

Laboratory of Physical and Structural Biology, NICHD, National Institutes of Health, Bethesda, MD 20892, USA

## ABSTRACT

General porin OmpF forms water-filled channels in the outer membrane of *E. coli* bacteria. When reconstituted into planar bilayer lipid membranes, these channels can be closed (or “gated”) by high electric fields. We discover that: (i) channel gating is sensitive to the type of cations in the membrane-bathing solution according to their position in the Hofmeister series; (ii) channel gates to a “closed” state that is represented by a set of multiple sub-conformations with at least three distinctly different conformations contributing to the closed-state conductance histogram. Taken together with the nearly symmetric response to the applied voltage of changing polarity and the hysteresis phenomena reported previously by others and reproduced here, these findings suggest that the voltage-induced closure of the OmpF channel is a consequence of reversible denaturation of the protein by the high electric field. If so, the voltage-induced gating of bacterial porins can serve as an instructive model to study the physics of protein folding at the single-molecule level.

**Keywords:** channel reconstitution, conductance fluctuations, hysteresis, Hofmeister series, single-molecule experiments

## 1. INTRODUCTION

OmpF (outer membrane protein F) is a protein that forms trimeric channels in the outer bacterial membrane<sup>1,2</sup>. The crystal structure of the trimer is known with 0.24 nm resolution<sup>3</sup>. Gram-negative bacteria like *Escherichia coli* are enclosed in a double membrane cellular envelope, and the outer membrane serves as a selective permeability barrier that protects bacteria from harmful components and acts as a passive filter regulating the flow of solutes. OmpF channels can be purified and reconstituted into planar lipid membranes to be studied there at the single-molecule level. Application of voltages exceeding about 100 mV causes these channels to spontaneously ‘gate’ to poorly conductive states.

The physiological role of voltage gating in general diffusion bacterial porins has been broadly debated over a long time (see, for example, corresponding chapters in the latest reviews<sup>1,2</sup> and references therein). On one hand, voltage gating phenomenon has been reproducibly observed by many groups using either planar lipid membrane technique (for the single channel current recordings see e.g. refs.<sup>4-6</sup>) or patch clamp electrophysiology<sup>7</sup>. On the other hand, the main evidence against a physiological role for the gating is that the critical voltage, above which the OmpF channels close appreciably, is larger than the naturally occurring potential across the outer membrane<sup>8</sup>.

This issue becomes even more complicated by the fact that the molecular mechanism for the voltage-dependent closure of porins is not known. Present-day hypotheses on the underlying molecular mechanism of voltage gating have been recently summarized in a minireview<sup>9</sup> (see also some discussions in refs.<sup>1,2</sup>). Briefly, the current concepts of structural basis of the gating phenomenon can be summarized as follows:

The *first* hypothesis suggests that motion of the L3 loop gates porins<sup>10,11,12</sup>. As soon as the first crystal structures of porins became available<sup>3</sup>, the L3 loop became a prime structural candidate for determining transport and gating characteristics of porins<sup>9</sup>. Particularly, studies of the atomic structures of bacterial porins with X-ray

crystallography<sup>3</sup> show that each monomer of this trimeric proteins is folded as a  $\beta$ -barrel with 16 antiparallel  $\beta$ -strands. Transmembrane strands are connected by short turns at the periplasmic side and by long loops at the external side. Unlike the other loops, the third loop, L3, folds into the barrel forming a constriction zone (also called the eyelet) approximately at half the height of the channel, which gives it an hourglass-like shape. At the constriction zone, a strong transverse electrostatic field is created by negatively charged (acidic) residues (Glu117 and Asp113) in L3 loop and a cluster of positively charged (basic) residues (Lys16, Arg42, Arg82, Arg132) at the opposite barrel wall. According to the hypothesis, high transmembrane voltages "gate" the OmpF channel by bringing the cationic and anionic amino acid side chains within the channel closer to each other<sup>1</sup>. However, when in subsequent experiments<sup>5</sup> the L3 loop was tethered to the barrel wall through disulfide bonds, the gating still occurred. This clearly ruled out the idea of at least large-scale movement of the L3 loop against the barrel wall.

The *second* concept is that specific residues inside the pore are responsible for the voltage gating in porins; it looks for explanation of the voltage gating phenomenon in screening - unscreening of charges in channel lumen. It has been shown<sup>13-15</sup> that mutations of charged residues in the L3 loop or on the facing barrel wall have a significant effect on the voltage gating of porins by changing so called "critical voltage". Thus, mutations R42C, R82C, and R132P lowered the critical voltage, whereas D113C, D113G, and E117C made the channel more stable under applied voltage. At the same time, some mutations that do not alter the net charge (Y102F and Y106F) have a strong effect on the critical voltage of OmpF<sup>15</sup>. Two other mutants (R42A/R82A/R132A/D113N/E117/Q and V18K/G131K) had the same critical voltage, even though one removed five charges and the other introduced two charges<sup>15</sup>. As far as we know, there are no published studies with mutants that are not sensitive to voltage.

According to the *third* scenario, motion of external loops that are located outside the channel gates the porin by folding back into the pore. This mechanism has been supported by an atomic force microscopy studies<sup>16</sup>, where direct imaging of OmpF crystals has revealed proteins in their normal and in what seemed to be a closed state with reversible changing between these conformations. In a recent paper on a homologous *Haemophilus influenzae* porin<sup>17</sup>, the idea of surface loops involvement was supported by site-directed mutagenesis.

The *forth* hypothesis examines voltage gating as an intrinsic property of  $\beta$ -barrels<sup>18</sup>. The authors draw our attention to the fact that several  $\beta$ -barrel toxins that do not have L3 loop or similar channel lumen constraining features still show typical porin-like voltage gating behavior in bilayer membranes (see, for example, ref.<sup>19</sup>). This concept considers voltage gating as a disruption of ion transport due to "perturbation of electric field in the pore"<sup>9,18</sup> by the applied voltage rather than some conformation changes of porin structure<sup>18</sup>.

In addition to the most commonly used hypotheses 1-4, authors of review<sup>9</sup> pointed out several other mechanisms. Such mechanisms might include unfolding and refolding of the porin, interaction with lipopolysaccharide molecules that might block pores (presumably, talking about unrefined samples), drastic movements of the porins inside the lipid matrix, and electrostriction effects on the membrane.

In the present study we first analyze these voltage-induced structural fluctuations and ion transport through the open channel (conductance and selectivity) along the series LiCl - NaCl - KCl - RbCl - CsCl. We find that the characteristic voltage of the channel transition to poorly conductive states depends on the type of alkali cations according to their position in the Hofmeister series. That is, it takes higher voltages to close the channel in LiCl than in CsCl. The channel conductance changes in this series in a manner that correlates with the solution conductivity only qualitatively, with Cs<sup>+</sup> cation being an exception. Our experiments show that the significant increase in channel conductance (about 2.5-fold from 0.1M LiCl to 0.1M RbCl) cannot be accounted for by just solution conductivity increase, even with the cationic selectivity of OmpF taken into account. Therefore, to explain both the cation-dependent channel structural sensitivity to the applied field and channel conductance, specific interactions between the cations and the channel have also to be considered.

Second, we prove that the poorly conductive state of the channel is represented by many protein conformations. We show the existence of at least three distinct levels of channel conductance in the "closed" state for each monomer in the channel trimer.

Based on these findings and results obtained by other authors who showed that the gating only weakly depends on the sign of the applied voltage and exhibits a significant hysteresis (see Fig. 4 in ref.<sup>2</sup>), we suggest that the voltage-induced closure of the OmpF channel can serve as a single-molecular model for studies of reversible protein denaturation by the high electric field.

We motivate this tentative conclusion by the fact that similar properties have been found for protein stability, folding, and denaturation<sup>20-25</sup>. In particular, the influence of different salts on the stability and pH-induced denaturation of the  $\alpha$ -helical membrane protein rhodopsin was found to follow the Hofmeister series<sup>20</sup>. Authors of this study report that kosmotropic anions favor a native or native-like fold of the protein while a non-native, denatured fold is favored in the presence of chaotropes. Sulfate anion stabilization of native ribonuclease A both by anion binding and by Hofmeister effect was observed in ref.<sup>21</sup>. The efficacies of several potassium salts and three chloride salts to stabilize the H2A-H2B histone dimer from the eukaryotic core nucleosome show that the Hofmeister effect is the predominant cause of stabilization<sup>23</sup>. Multiple unfolded states were described for alcohol dehydrogenase<sup>24</sup> and for immunoglobulin domain of the human cardiac muscle protein titin<sup>25</sup>.

## 2. MATERIALS AND METHODS

“Solvent-free” planar DPhPC lipid bilayers were formed using lipid monolayer opposition technique from 5 mg/ml solution of DPhPC in pentane on a 60  $\mu$ m diameter aperture in the 15  $\mu$ m thick Teflon film that separated two (cis and trans) compartments of the Teflon chamber as previously described<sup>26</sup>. The aperture was pretreated with 1% solution of hexadecane in n-pentane and dried during 15 min prior membrane formation. The film and the total capacitances were close to 25 and 50 pF, respectively.

The electrical potential difference across the membrane was applied with a pair of Ag-AgCl electrodes in 2M KCl, 1.5% agarose bridges. Potential was determined as positive when it was greater at the side of OmpF addition (cis-side). The signal amplification and analysis techniques were as described previously<sup>27</sup>. All experiments were carried out at room temperature of  $23 \pm 2^\circ\text{C}$ .

Wild type OmpF was a generous gift of Dr. Mathias Winterhalter. Single channels were formed by adding  $(0.1 \pm 0.3) \mu\text{l}$  of 1  $\mu\text{g/ml}$  stock solution of OmpF to 1.5 ml aqueous phase in the cis half of the chamber while stirring at  $-(180 \pm 200)$  mV of applied voltage for approximately 5 min. LiCl, NaCl, KCl, CsCl and RbCl solutions were buffered by MES or HEPES with a final concentration of 5 mM. Stock solution pH was adjusted by adding Li(Na,K,Cs, Rb)OH or HCl. Control experiments at pH 5  $\div$  pH 6 in the absence of any buffers showed no dependence of OmpF properties (conductance and open channel noise) on the buffer nature as well as on the presence of buffer. Solution conductivities (**Table 1**) were measured using a CDM 83 conductivity meter (Radiometer, Copenhagen, Denmark) at  $23.0^\circ\text{C}$ .

Table 1. Solution conductivities		
$\lambda$ , mS/cm	0.1 M	1M
LiCl	9.15	68.7
NaCl	10.25	80.5
KCl	12.42	104.5
CsCl	12.75	107.2
RbCl	12.88	108.2

Multichannel bilayer experiments were performed on a 120  $\mu$ m-diameter aperture that gave the total membrane capacitance of about 200 pF. In this case, channels were formed by adding of 10-20  $\mu\text{l}$  of 20  $\mu\text{g/ml}$  stock solution of OmpF to the bathing solution in the cis compartment of the chamber. The steady macroscopic conductance was achieved in about one hour.

The cation-anion selectivity was analyzed by measuring zero current potential,  $E_{rev}$ , under conditions of a ten-fold gradient of KCl concentration across the bilayer. The cationic transport number ( $t_K^+$ ), related to the anionic transport number ( $t_{Cl^-}$ ) by  $t_K^+ + t_{Cl^-} = 1$ , was calculated using

$$E_{rev} = (1 - 2t^+) \frac{kT}{e} \ln \frac{a_{1M}}{a_{0.1M}},$$

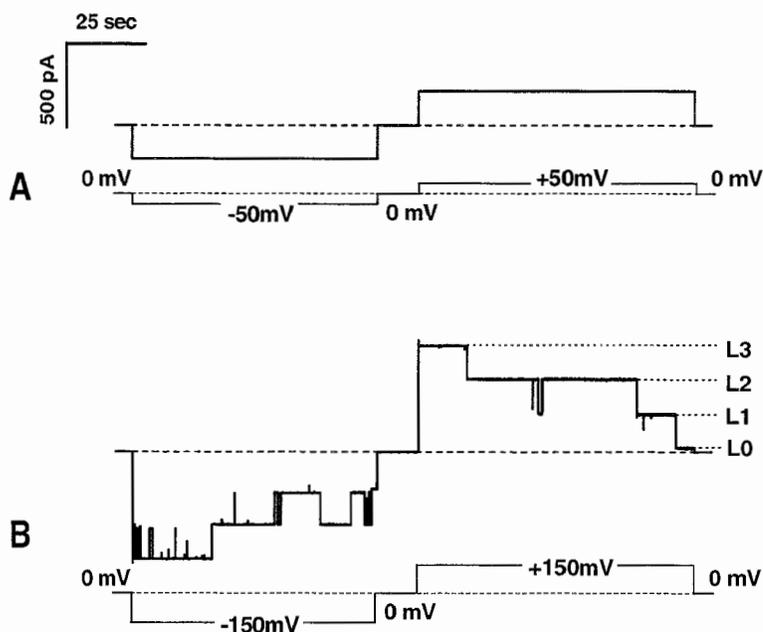
where  $a_{1M}$  and  $a_{0.1M}$  are salt activities respectively in 1M and 0.1 M solutions and  $k$ ,  $T$ , and  $e$  have their usual meaning of the Boltzmann constant, the absolute temperature, and the electron charge.

### 3. RESULTS AND DISCUSSION

In the earliest studies when the structure of porins was completely unknown, the observation of gating inspired some scientists to propose that porins might serve as "models" of voltage-gated ion channels in nerve-cells (see ref.<sup>1</sup>). Traditionally, "voltage gating" implies the switching of ion current through transmembrane channels under applied voltage. This clearly occurs in the voltage-dependent sodium, potassium and calcium channels, which combine to create the action potential<sup>18</sup>. In each case the channel respond to changes in the transmembrane potential difference by altering their probability of opening. These voltage-gated channels of electrophysiology are constructed in a totally different manner from the  $\beta$ -barrel pores we discuss here.

The voltage gating in porins possesses the following typical features (see current tracks in Fig. 1):

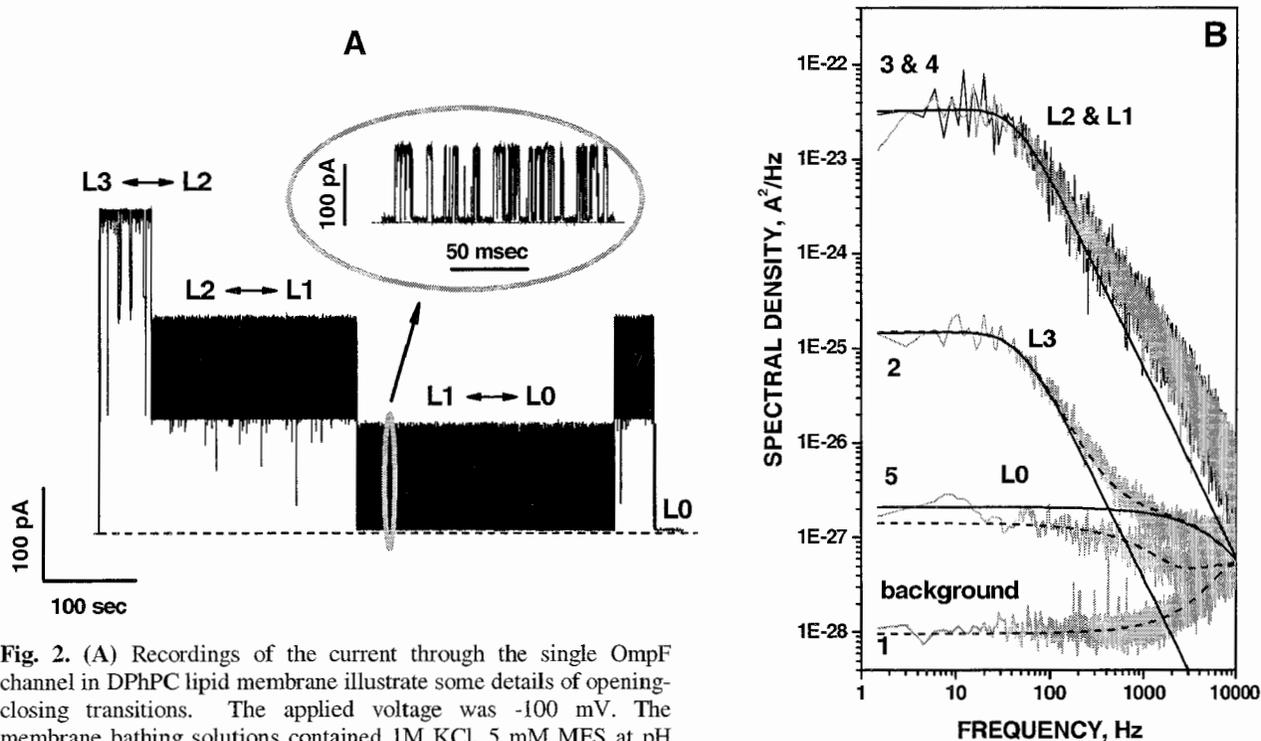
- Being in a few cases slightly asymmetrical, voltage gating in porins always occurs at both positive and negative applied potentials (Fig. 1B). This is unusual for the  $\alpha$ -helical channels of electrophysiology that indeed respond to polarity changes<sup>18</sup>;
- At relatively low voltages, porins can be stabilized in their open state for seconds and even minutes;
- Closed states of the porins usually last for seconds or minutes while the switching time for e.g. sodium channels is of the order of milliseconds<sup>18</sup>;



**Fig. 1.** Typical recordings of ion current through single trimeric OmpF channel reconstituted into planar lipid bilayer membranes at  $\pm 50$  mV (A) and  $\pm 150$  mV (B) of applied voltage. It is seen (B), that large voltages close the channel in three approximately equal but not totally identical steps, revealing its trimeric organization. The dashed lines show zero current levels, the dotted lines in Fig. 1B designate a fully open state (L3, all three monomers open) and two partially closed states (L2, two monomers open; L1, one monomer open). Note the presence of residual conductance (L0) after the total three-step closure. Time resolution was 10 msec, the membrane bathing solutions contained 1M KCl, 5 mM HEPES at pH 7.4.

- Being once closed, one, two or all three monomers of the porin quite often (but not necessarily) remain in their closed state even when transmembrane voltage is switched to 0 mV or low “non-gating” voltages. It is interesting that the probability to find porins in this state increases with the time channel is kept in its closed state under the high applied voltage;
- Surprisingly, applying high voltage of opposite size (e.g. -150 mV vs. 150 mV and vice versa), that normally causes gating in porins too, promotes reopening;
- Occasionally, fast (noise-like) flickering between open and closed states of individual monomers can be recorded (**Fig. 2**);
- As voltage is brought down, channel re-opening shows a distinct and characteristic hysteresis both for multi-channel (**Fig. 3**) and single-channel membranes. This phenomenon for porins has been first seen by Schindler and Rosenbusch<sup>28</sup> and studied later in refs.<sup>29,30</sup>. Note, however, that hysteretic-like behaviors have been also reported for voltage-activated cation channel in the human red blood cell membrane in ref.<sup>31</sup> (see also a simple model for it in ref.<sup>32</sup>), HERG-like K<sup>+</sup> channels from microglia<sup>33,34</sup>, and for N-methylaspartate channels<sup>35</sup>.

It is remarkable that several other  $\beta$ -barrel-like pores, e.g. aerolysin (hemolysin from *Aeromonas hydrophila*<sup>36</sup>),  $\alpha$ -hemolysin from *Staphylococcus aureus*<sup>37-40</sup>, and the voltage-dependent anion channel (VDAC) family or “mitochondrial porins”<sup>41,42</sup> share many of the features of voltage gating listed above (T. K. Rostovtseva, O. Krasilnikov personal communications). This resemblance taken together with the current knowledge of porin and pore-forming toxin structures supports the idea<sup>18</sup> that voltage gating is a fundamental feature of porin and toxin  $\beta$ -barrel membrane channels. However, the gating peculiarities shortly described above appear to be too complex to be explained simply by voltage-dependent “breakdown of the delicate ion conducting pathway”<sup>18</sup>.

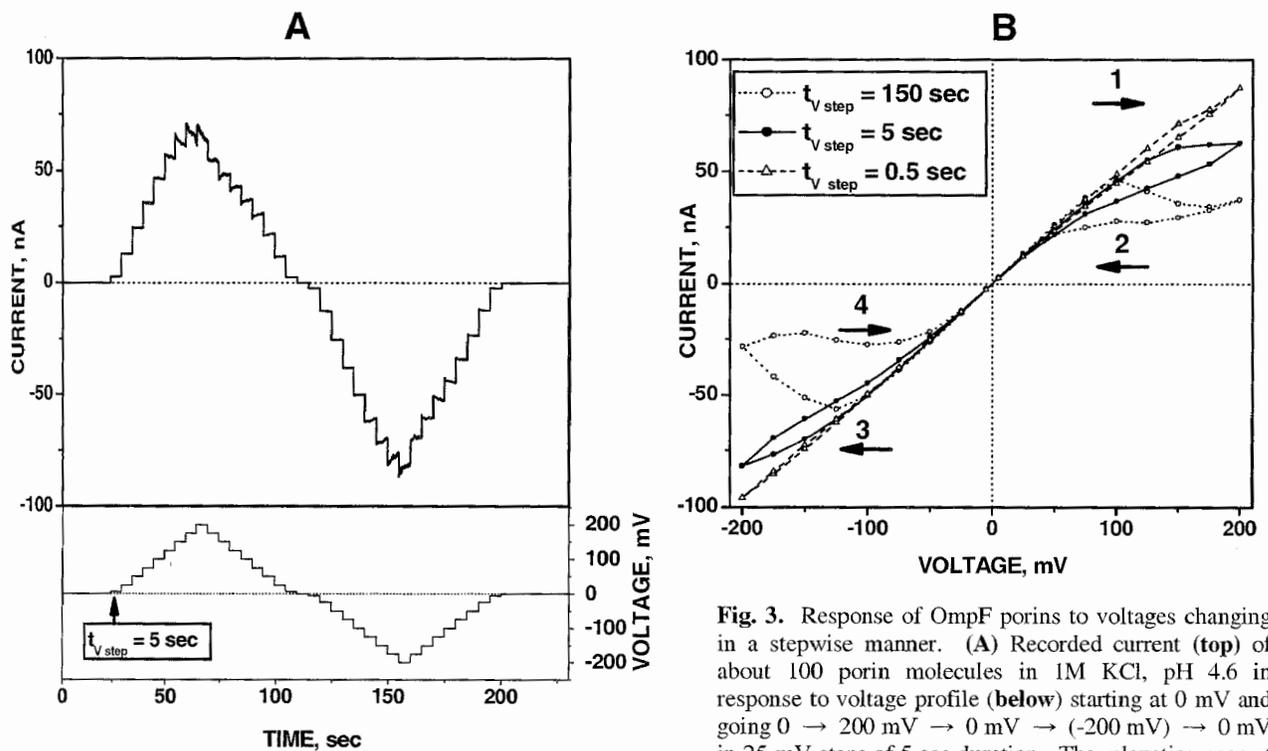


**Fig. 2.** (A) Recordings of the current through the single OmpF channel in DPhPC lipid membrane illustrate some details of opening-closing transitions. The applied voltage was -100 mV. The membrane bathing solutions contained 1M KCl, 5 mM MES at pH 3.9. Time resolution was 10 msec for the main recording and 1 msec for the inset.

(B) Power spectral density of noise in the current through a single OmpF channel shown in panel (A). Background spectrum (curve 1) was measured for the membrane with a single OmpF channel at 0 mV. Curve 2 corresponds to noise of the fully open channel (the nature of the Lorentzian component as well as of the high-frequency noise for this curve has been discussed in our recent paper<sup>6</sup>). Curves 3 and 4 represent fast flickering between levels L2 and L1 and between levels L1 and L0, correspondingly. The equivalence of these two curves suggests that only one of the three porin monomers is involved in fast flickering between its open and closed state. At  $f < 300$  Hz the spectra of low-pH fluctuations can be approximated by single Lorentzians with characteristic time of about 3 msec. Dashed lines through the spectra were drawn by eye.

In this study we give support to the gating hypotheses that consider closing of these large-diameter pores as some large-scale structural changes (e.g., reversible denaturation) of  $\beta$ -barrels. This idea has been supported most strongly by studies with VDAC channel whose "voltage sensor" is believed to be formed by the part of the wall of the channel in the open state. Upon channel closure this part is moved out of the channel (for review see ref.<sup>43</sup>). The open and closed VDAC channel conformations differ not only in conductance but also in selectivity. The open state prefers anions, e.g.,  $\text{Cl}^-$  over  $\text{K}^+$  by a factor of 5 at 0.1 M salt concentration. The "closed states" (multiple less conductive states) generally favor cations. In addition, probing  $\alpha$ -toxin channel with water soluble polymers yields the values of apparent radii of  $\sim 1.3$  nm and  $\sim 0.9$  nm, respectively, for the open and closed (but still conductive with >10-fold difference) conformations<sup>44</sup>.

Interpreting voltage gating as a result of reversible but significant structural changes in the protein open conformation, one might expect to see the dependence of this gating on solution composition in terms of Hofmeister effect. In his original work in 1888, Hofmeister composed the series for cations and anions by ordering various ions in respect of their ability to influence protein solubility.



**Fig. 3.** Response of OmpF porins to voltages changing in a stepwise manner. (A) Recorded current (top) of about 100 porin molecules in 1M KCl, pH 4.6 in response to voltage profile (below) starting at 0 mV and going  $0 \rightarrow 200 \text{ mV} \rightarrow 0 \text{ mV} \rightarrow (-200 \text{ mV}) \rightarrow 0 \text{ mV}$  in 25-mV steps of 5 sec duration. The relaxation seen at high voltages is due to the closure of OmpF channels. The entire experiment was performed on a single DPhPC

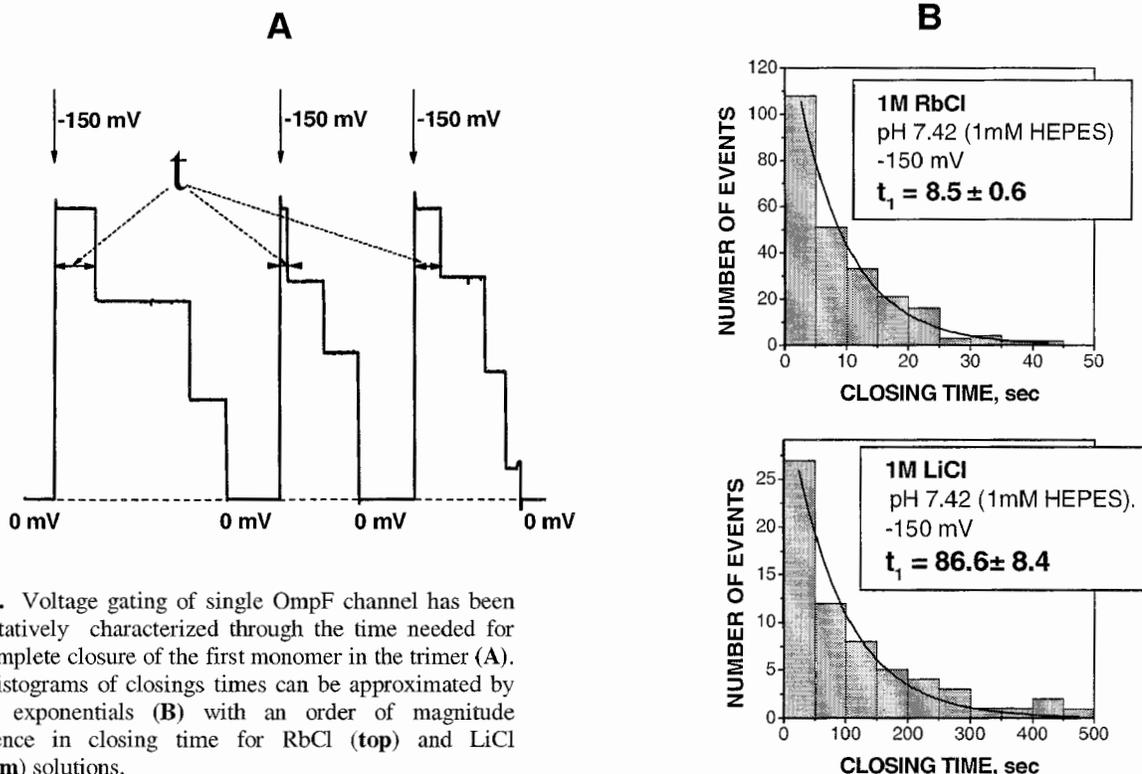
membrane. Time resolution was 10 msec. (B) *I-V* diagrams illustrate hysteresis in porin behavior in response to changing voltage. Three recordings, different in the rate of voltage change, were taken from the same OmpF-containing membrane: 150 sec per 25-mV voltage step (*open circles*), 5 sec per 25-mV voltage step (*filled circles*), and 0.5 sec per 25-mV voltage step (*open triangles*). Note that the data represented by closed circles were obtained directly from the current recording shown in (A). Current amplitudes were read at the very end of each voltage step. It is seen that the deviation of the current trace from linearity is minimal for the curve with 0.5 sec per voltage step recording; the measurements were performed too fast to allow OmpF channels to reach their closed state. Arrows indicate the direction of the sequence in which the points were measured. Solid lines are drawn by eye.

An impressive number of later works show, that with minor exception, the same Hofmeister series emerge in studies of denaturation, depolymerization, and dissociation of proteins, and of inhibition or activation of enzymes (e.g., see refs.<sup>45-47</sup>). Nowadays the Hofmeister series are often given in terms of the ability of the ions to stabilize the structure of proteins. Since generally OmpF selectivity is preferentially cationic (with a factor of 3), here we examine the influence of monovalent cations:  $\text{Li}^+$ ,  $\text{Na}^+$ ,  $\text{K}^+$ ,  $\text{Rb}^+$ , and  $\text{Cs}^+$  on OmpF stability. To find possible parallels with the influence on voltage gating, we also analyze single OmpF channel selectivity and conductance along this cation series.

Voltage gating in similar systems has been traditionally studied in multi-channel bilayer lipid membranes by measuring "critical voltage"<sup>5</sup> ( $V_c$ ). **Table 2** contains the data for critical voltages obtained in presence of different cations from the Hofmeister series as bathing electrolytes. It is seen, that in LiCl solution, critical voltages are high and channels are stabilized in their open conformation, whereas in CsCl solutions closed states are more easily reached. For the OmpF-containing membranes, this method has definite disadvantages. For example, scatter of data from experiment to experiment, namely, from one multi-channel membrane to another is too high, often of the same order that the effect produced by changing cations itself.

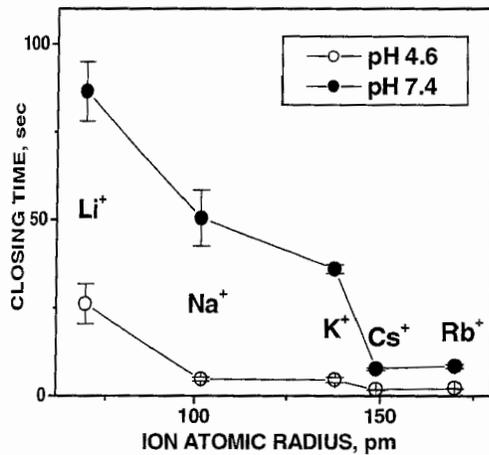
Electrolyte	LiCl	NaCl	KCl	CsCl	RbCl
$V_c$ in 1M, mV	$150 \pm 9$	$138 \pm 8$	$131 \pm 7$	$128 \pm 8$	$115 \pm 8$
$V_c$ in 0.1M, mV	$128 \pm 8$	$124 \pm 9$	$115 \pm 8$	$113 \pm 8$	$106 \pm 8$

For this reason we propose to examine the stability of *single* porin channels in bilayer lipid membranes in response to high transmembrane voltages in various experimental conditions. The method is illustrated in **Fig. 4**. We measure the time needed for the closure of one (first) monomer in the trimer under  $-150$  mV of applied voltage. After each measurement  $0$  mV is applied to reopen the channel. Examination of Fig. 4A shows that the time spent in the completely open conformation after the voltage is applied (closing time) is a random variable. Indeed, similarly to other channels, voltage gating of OmpF is a random process.



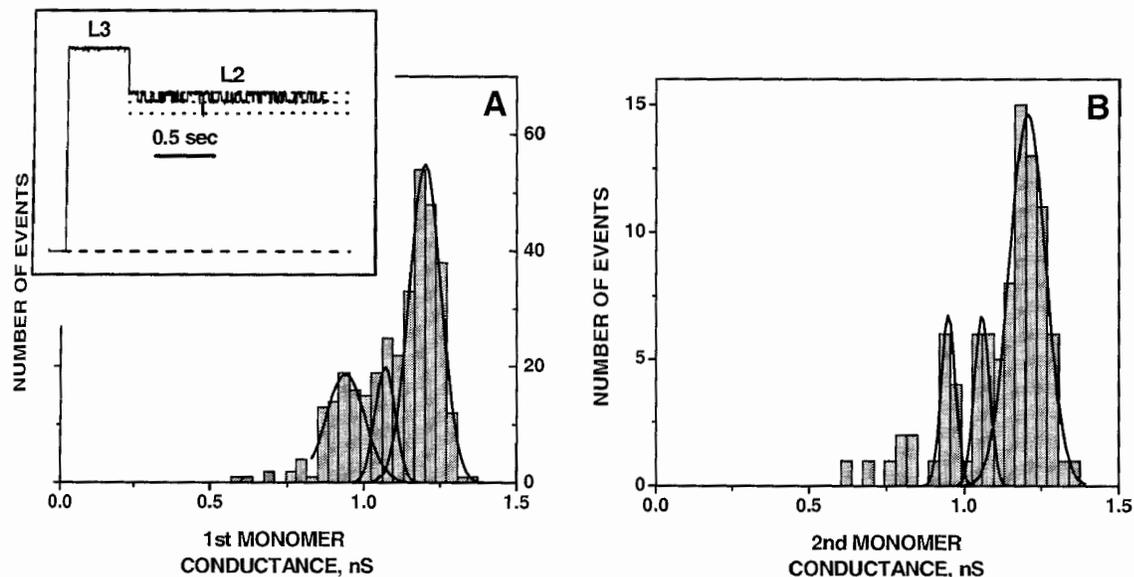
**Fig. 4.** Voltage gating of single OmpF channel has been quantitatively characterized through the time needed for the complete closure of the first monomer in the trimer (A). The histograms of closings times can be approximated by single exponentials (B) with an order of magnitude difference in closing time for RbCl (top) and LiCl (bottom) solutions.

It means that to measure the average closing time accurately, we have to gather enough statistics. In each single-channel experiment the average closing time was calculated from the exponential fitting of a time histogram obtained from many repeated measurements illustrated in Fig. 4A. **Figure 4B** illustrates the dramatic difference (about 10 times) in closing time for RbCl and LiCl bathing solutions.



**Fig. 5.** Closing times of a single OmpF channel in the series Cs<sup>+</sup>, Rb<sup>+</sup>, K<sup>+</sup>, Na<sup>+</sup>, Li<sup>+</sup> at pH 4.6 (open circles) and pH 7.4 (filled circles). OmpF gates more easily in the presence of ions with large atomic radii (and less degree of hydration) such as Rb<sup>+</sup> and Cs<sup>+</sup>. In the presence of highly hydrated Li<sup>+</sup> the open conformation of the channel is more stable. The membrane bathing solutions contained 1M of Li(Na, K, Cs, Rb)Cl, 5 mM MES for pH 4.6 and 5 mM HEPES for pH 7.4. The applied voltage was -150 mV.

**Figure 5** summarizes our results on closing times for all studied cations for two different pH values, namely pH 4.6 and pH 7.4. It is seen that the time needed to close a single pore is decreasing with the increasing atomic radius of cations. In other words, it is clear that ions with large atomic radii and weak interaction with water (so called chaotropes) such as Rb<sup>+</sup> and Cs<sup>+</sup> greatly promote the closure of an open OmpF channel. Ions with small atomic radii, such as Li<sup>+</sup>, that strongly interact with water (kosmotropes) show significantly longer closing time.

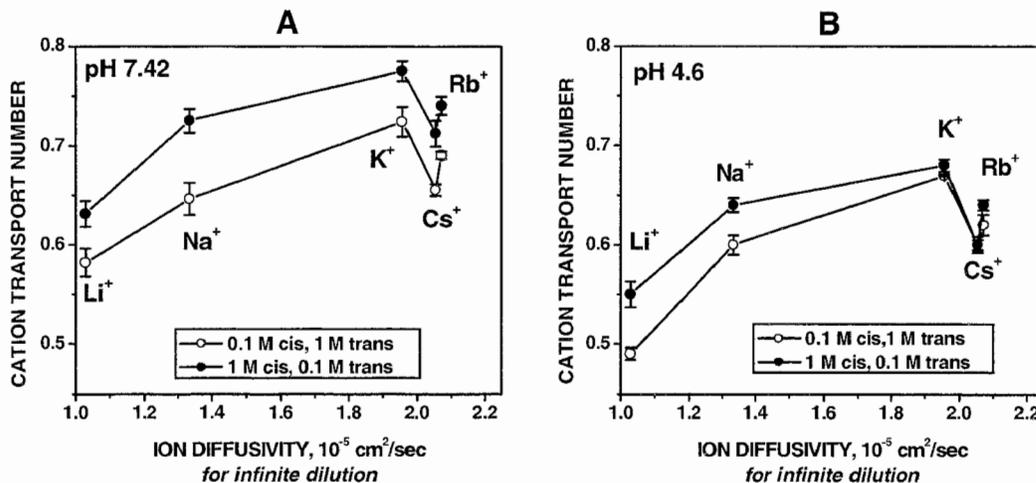


**Fig. 6** Conductance histograms of the voltage gating events measured for the same single OmpF channel under applied -150 mV in 1M CsCl solution at pH 7.4 (5 mM HEPES). **(A)** Closing events for the 1<sup>st</sup> monomer in the trimer. **(B)** Only those 2<sup>nd</sup> monomer closing events were selected for this panel that followed large steps in the 1<sup>st</sup> monomer closing (peak #2 with 1.07 nS conductance and peak #3 with 1.2 nS conductance in panel (A)). Three peaks on each curve correspond to three clearly defined conductance sub-states. The amplitudes of these peaks are equal to  $(0.94 \pm 0.02)$  nS,  $(1.07 \pm 0.01)$  nS,  $(1.2 \pm 0.01)$  nS for (A), and  $(0.95 \pm 0.01)$  nS,  $(1.06 \pm 0.01)$  nS,  $(1.21 \pm 0.01)$  nS for (B). **Inset** in panel (A) shows fluctuations between single monomer closed conformation sub-states.

As we mentioned above, OmpF closure is never complete. Each downward step induced by the applied voltage is slightly smaller than one third of the total conductance of the fully open channel. After all three monomers are "closed", the residual conductance amounts to 2-5% of the initial conductance. In **Fig. 6A** we plotted an amplitude histogram of the conductance steps corresponding to the closure of the first monomer in the trimer (see Fig. 4A). At least three well-defined peaks with about 10% differences between conductances can be easily seen in the histogram. In **Fig. 6B** we prove that these peaks are not an attribute of trimeric OmpF organization but intrinsic properties of voltage gating of *each monomer*. Indeed, when only those events that followed the large steps in the 1<sup>st</sup> monomer closure (corresponding to the 2<sup>nd</sup> and 3<sup>rd</sup> peaks in Fig. 6A) were selected we still observe three-peak histogram. Here we propose that the three peaks correspond to three clearly defined sub-states of the closed conformation of each OmpF monomer. Moreover, in some experiments we could observe noise-like fast transitions in the current between the two (or even all three) closed conformations of the protein (see Fig. 6A *inset*). Interestingly, amplitudes of these transitions were equal to the differences between the peaks in Fig. 6.

It is generally accepted that OmpF shows a slight selectivity to cations, namely prefers cations over anions with a factor of three. Recently<sup>6</sup>, we have shown that this is true only for pH values close to neutral. By varying the solution pH we could change this preference in favor of anionic selectivity in low pH solutions. In **Fig. 7** we collected the OmpF selectivity data from 1M and 0.1M solutions of LiCl, NaCl, KCl, CsCl and RbCl for pH 7.4 and 4.6. It is seen that OmpF selectivity significantly depends on the type of bathing electrolyte that surrounds the membrane. In a certain sense, OmpF can be called a potassium channel since it shows the highest cationic selectivity in case of KCl bathing solution for both studied pH values. In LiCl solutions OmpF is hardly cationic selective, since transport numbers for cations and anions are quite close even at neutral pH. It is interesting to note that OmpF selectivity obeys the 4<sup>th</sup> Eisenman sequence for equilibrium ion exchange.

**Figure 8** shows conductance of a single OmpF channel as a function of voltage in 0.1 and 1 M solutions of the salt used in this study. It is seen that an asymmetry of channel these characteristics strongly depends on the bathing solution concentration and electrolyte type. At high concentrations the conductance curves is practically voltage-independent. However, in 0.1 M RbCl and 0.1 M LiCl solutions conductance of the channel is higher at -200 mV than at +200 mV by 35% and 24%, respectively. It is interesting that highly hydrated and less mobile Li<sup>+</sup> is less sensitive to the asymmetry of the open OmpF channel. Note, that even though the bulk conductivities of CsCl solutions exceed those of KCl (Table 1), the OmpF conductance is larger with KCl. This agrees with our selectivity data (Fig. 7) that show higher selectivity of OmpF to potassium ions.



**Fig. 7.** OmpF selectivity along the series Li<sup>+</sup>, Na<sup>+</sup>, K<sup>+</sup>, Rb<sup>+</sup>, Cs<sup>+</sup> measured under two oppositely directed salt gradients: 0.1M cis / 1 M trans (*open circles*), 1M cis / 0.1M trans (*filled circles*) at pH 7.4 (A) and pH 4.6 (B). OmpF is more selective for cations when the more concentrated solution is added to the cis side of the chamber in comparison with the opposite solutions gradient. This finding most likely reflects an asymmetry of charge distribution in the OmpF molecule (A. Alcaraz, E. M. Nestorovich, M. Aguilera-Arzo, V. M. Aguilera, S. M. Bezrukov, manuscript in preparation).

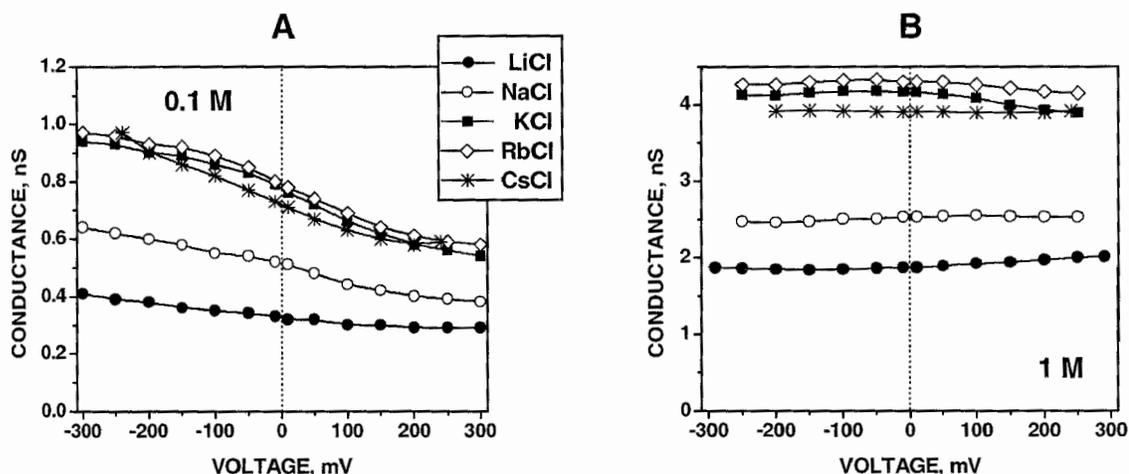


Fig. 8. Asymmetry of channel conductance strongly depends on the bathing solution concentration and electrolyte type for both 0.1M (A) and 1M (B) solutions.

## CONCLUSIONS

Our main findings are:

- According to their position in the Hofmeister series, different species of alkali metal cations destabilize the fully open conformation of the OmpF channel to a different degree;
- The poorly conductive “closed” state of the channel is represented by multiple conformations with at least three sub-conformations contributing to the conductance histogram of each monomer in the channel trimer.

Combined with the previously obtained results on gating hysteresis and on nearly symmetrical gating response to the electric fields of opposite polarities, our study suggests that the voltage-induced closure of bacterial porins is a manifestation of reversible protein denaturation by high electric fields. If so, the “gating” of bacterial porins by voltage can serve as an informative model for studying protein denaturation and re-folding back to completely functional states at the single-molecule level. Experiments of this kind are able to advance our understanding of the kinetics and energies involved in the late stages of protein folding<sup>48,49</sup>.

## REFERENCES

1. H. Nikaïdo, “Molecular basis of bacterial outer membrane permeability revisited”, *Microbiol. Mol. Biol. Rev.*, **67**, 593-556, 2003.
2. A. H. Delcour, “Solute uptake through general porins”, *Front. Biosci.*, **8**, d1055-71, 2003.
3. S. W. Cowan, T. Schirmer, G. Rummel, M. Steiert, R. Pauptit, R. A. Jansonius, and J. P. Rosenbusch, “Crystal structure explains functional properties of two *E.coli* porins”, *Nature*, **358**, 727-733, 1992.
4. J. Schindler, and J. P. Rosenbusch, “Matrix protein from *E.coli* outer membrane forms voltage-controlled channels in lipid bilayers”, *Proc. Natl. Acad. Sci. U.S.A.*, **75**, 3751-3755, 1978.
5. G. Bainbridge, H. Mobasher, G. A. Armstrong, E. J. A. Lea, and J. H. Lakey. “Voltage-gating of *E.coli* porin: a cysteine-scanning mutagenesis study of loop 3”, *J. Mol. Biol.*, **275**, 171-176, 1998.
6. E. M. Nestorovich, T. K. Rostovtseva, and S. M. Bezrukov, “Residue ionization and ion transport through OmpF channels”, *Biophys. J.*, **85**, 3718-3729, 2003.

7. A. H. Delcour, B. Martinac, C. Kung, and J. Adler, "Voltage-sensitive ion channel of *E.coli*", *J. Memb. Biol.*, **112**, 267-275, 1989.
8. K. Sen, J. Hellman, and H. Nikaido, "Porin channels in intact cells of *E.coli* are not affected by Donnan potentials across the outer membrane", *J. Biol. Chem.*, **263**, 1182-1187, 1988.
9. K. M. Robertson, and D. P. Tieleman, "Molecular basis of voltage gating of OmpF porin", *Biochem. Cell. Biol.*, **80**, 517-523, 2002.
10. M. Brunen, and H. Engelhardt, "Asymmetry of orientation and voltage gating of the Acidovarax delafieldii porin Omp34 in lipid bilayers", *Eur. J. Biochem.*, **212**, 129-135, 1993.
11. M. Watanabe, J. Rosenbusch, T. Schirmer, and M. Karplus, "Computer simulations of the OmpF porin from the outer membrane of *E.coli*", *Biophys. J.*, **72**, 2094-2102, 1997.
12. C. M. Soares, J. Björksen, and O. Tapia, "L3 loop-mediated mechanisms of pore closing in porin: a molecular dynamics perturbation approach", *Protein Eng.*, **8**, 5-12, 1995.
13. P. Van Gelder, N. Saint, P. Phale, E. F. Eppens, A. Prilipov, R. van Boxtel, and J. P. Rosenbusch, "Voltage sensing in the PhoE and OmpF outer membrane porins in of *E.coli*: role of charged residues", *J. Mol. Biol.*, **269**, 468-472, 1997.
14. J. Bredin, N. Saint, M. Malléa, E. De, G. Molle, J. - M. Pagés, and V. Simonet, "Alteration of pore properties of *E.coli* OmpF induced by mutation of key residues in anti-loop 3 region", *Biochem. J.*, **363**, 521-528, 2002.
15. P. S. Phale, A. Philippsen, C. Widmer, V. P. Phale, J. P. Rosenbusch, and T. Schirmer, "Role of charged residues at the OmpF porin channel constriction probed by mutagenesis and simulation", *Biochemistry*, **40**, 6319-6325, 2001.
16. D. J. Müller, and A. Engel, "Voltage and pH-induced channel closure of porin OmpF visualized by atomic force microscopy", *J. Mol. Biol.*, **285**, 1347-1351, 1999.
17. M. A. Arbing, J. W. Hanrahan, and J. W. Coulton, "Mutagenesis identifies amino acid residues in extracellular loops and within the barrel lumen that determine voltage gating of porin from *Haemophilus influenzae* type b", *Biochemistry*, **40**, 14621-14628, 2001.
18. G. Bainbridge, I. Gokce, and J. H. Lakey, "Voltage gating is a fundamental feature of porin and toxin  $\beta$ -barrel membrane channels", *FEBS. Lett.* **431**, 305-308, 1998.
19. H. U. Wilmsen, F. Pattus, J. T. Buckley, "Aerolysin, a hemolysin from *Aeromonas hydrophila*, forms voltage-gated channels in planar lipid bilayers", *J Membr Biol.*, **115**, 71-81, 1990.
20. R. Vogel, and F. Siebert, "Conformation and stability of  $\alpha$ -helical membrane proteins.2. Influence of pH and salts on stability and unfolding of rhodopsin", *Biochemistry*, **41**, 3536-3545, 2002.
21. C. H. I. Ramos, R. L. Baldwin, "Sulfate anion stabilization of native ribonuclease A both by anion binding and by Hofmeister effect", *Protein Sci*, **11**, 1771-1778, 2002.
22. A. C. Apetri, and W. K. Surewicz, Atypical effect of salts on the thermodynamic stability of human prion protein", *J. Biol. Chem.*, **278**, 22187-22192, 2003.
23. L.M. Gloss, and B. J. Placek, "The effect of salts on the stability of the H2A-H2B histone dimer", *Biochemistry*, **41**, 14951-14959, 2002.
24. P. Sacchetta, R. Di Rado, M. Saliola, A. Bozzi, C. Falcone, C. Di Ilio, and F. Martini, "Multiple unfolded states of alcohol dehydrogenase I from *Kluyveromyces lactis* by guanidinium chloride", *Biochim. Biophys. Acta*, **1545**, 238-244, 2001.
25. C. F. Wright, K. Lindorff-Larsen, L. G. Randles, and J. Clarke, "Parallel protein-unfolding pathways revealed and mapped", *Nat. Struct. Biol.*, **10**, 658-662, 2003.
26. S. M. Bezrukov, and I. Vodyanoy, "Probing alamethicin channels with water-soluble polymers. Effect on conductance of channel states", *Biophys. J.*, **64**, 16-25, 1993.
27. T. K. Rostovtseva, E. M. Nestorovich, and S. M. Bezrukov, "Partitioning of differently sized poly(ethylene glycol)s into OmpF porin", *Biophys. J.*, **82**, 160-169, 2002.
28. H. Schindler, and J. P. Rosenbusch, "Matrix protein in planar membranes: clusters of channels in a native environment and their functional reassembly", *Proc. Natl. Acad. Sci. U.S.A.*, **78**: 2302-2306, 1981.
29. M. Simon, A. Mathes, A. Blanch, and H. Engelhardt, "Characterization of a porin from the outer membrane of *Vibrio anguillarum*", *J.Bacteriol.*, **178**, 4182-4188, 1996.
30. A. Mathes, and H. Engelhardt, "Voltage-dependent closing of porin channels: analysis of relaxation kinetics", *J. Membrane Biol.*, **165**, 11-18, 1998.

31. L. Kaestner, P. Christophersen, I. Bernhardt, P. Bennekou, "The non-selective voltage-activated cation channel in the human red blood cell membrane: reconciliation between two conflicting reports and further characterization", *Bioelectrochemistry*, **52**, 117-125, 2000.
32. E. Gudowska-Nowak, H. Flyvbjerg, P. Bennekou, and P. Christophersen, "Hysteresis in channel gating", *Unsolved Problems of Noise and Fluctuations: UPoN: Third International Conference*, 305-311, 2002.
33. P. S. Pennefather, W. Zhou, T. E. DeCoursey, "Idiosyncratic gating of HERG-like K<sup>+</sup> channels in microglia", *J Gen Physiol*, **111**, 795-805, 1998.
34. W. Zhou, F. S. Cayabyab, P. S. Pennefather, L. C. Schlichter, T. E. DeCoursey, "HERG-like K<sup>+</sup> channels in microglia". *J Gen Physiol*, **111**, 781-794, 1998.
35. L. M. Nowak, J. M. Wright, "Slow voltage-dependent changes in channel open-state probability underlie hysteresis of NMDA responses in Mg(2+)-free solutions", *Neuron*, **8**, 181-187, 1992
36. G. Menestrina, "Ionic channel formed by *S. aureus*  $\alpha$ -toxin: voltage-dependent inhibition by divalent and trivalent cations", *J. Membr. Biol.*, **90**, 177-190, 1986.
37. G. Menestrina, "Pore formation by *Staphylococcus aureus* alpha-toxin: a study using planar bilayers", *Zbl. Bakteriol. Suppl.*, **17**, 295-302, 1988.
38. O. V. Krasilnikov, P. G. Merzliak, R. Z. Sabirov, and B. A. Tashmukhamedov, "Memory is a property of an ion channels pool: ion channels formed by *Staphylococcus aureus* alpha-toxin", *Gen. Physiol. Biophys.*, **9**, 569-575, 1990.
39. J. J. Kasianowicz and S. M. Bezrukov, "Protonation dynamics of the alpha-toxin ion channel from spectral analysis of pH-dependent current fluctuations", *Biophys. J.*, **69**, 94-105, 1995.
40. Y. E. Korchev, C. L. Bashford, C. M. Alder, J. J. Kasianowicz and C. A. Pasternak, "Low conductance states of a single ion channel are not closed", *J. Membr. Biol.*, **147**, 233-239, 1995.
41. M. Colombini, "Voltage gating in the mitochondrial channel, VDAC", *J. Membr. Biol.*, **111**, 103-111, 1989.
42. S. J. Schein, M. Colombini, and A. Finkelstein, "Reconstitution in planar lipid bilayers of a voltage-dependent anion-selective channel obtained from *Paramecium* mitochondria", *J. Membr. Biol.*, **30**, 99-120, 1976.
43. M. Colombini, E. Blachli-Dyson, and M. Forte, "VDAC, a channel in the outer mitochondrial membrane", *Ion channels*, volume **4**, edited by Toshio Naraschi, Plenum Press, New York, ch. 5, 169-202, 1996.
44. O. V. Krasilnikov, P. G. Merzlyak, L. N. Yuldasheva, and R. A. Nogueira, "Channel-sizing experiments in multichannel bilayers", *Gen. Physiol. Biophys.*, **17**, 349-363, 1998.
45. A. Neagu, M. Neagu, and A. Der, "Fluctuations and the Hofmeister effect", *Biophys. J.*, **81**, 1285-1294, 2001.
46. K. D. Collins, and M. W. Washabaugh, "The Hofmeister effect and the behavior of water at interfaces", *Q. Rev. Biophys.*, **18**, 323-422, 1985.
47. M. G. Cacace, E. M. Landau, and J. J. Ramsden, The Hofmeister series: salt and solvent effects on interfacial phenomena, *Q. Rev. Biophys.*, **30**, 241-278, 1997.
48. H. Frauenfelder, S. G. Sligar, and P. G. Wolynes, "The energy landscapes and motions of proteins", *Science*, **254**, 1598-1603, 1991.
49. H. Frauenfelder, and D. T. Leeson, "The energy landscapes in non-biological and biological molecules", *Nat. Struct. Biol.*, **5**, 757-759, 1998.