Overcharging below the nanoscale: Multivalent cations reverse the ion selectivity of a biological channel

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We report charge inversion within a nanoscopic biological protein ion channel in salts of multivalent ions. The presence of positive divalent and trivalent counterions reverses the cationic selectivity of the OmpF channel, a general diffusion porin located in the outer membrane of *E. coli*. We discuss the conditions under which charge inversion can be inferred from the change in sign of the measured quantity, the channel zero current potential. By comparing experimental results in protein channels whose charge has been modified after site-directed mutagenesis, the predictions of current theories of charge inversion are critically examined. It is emphasized that charge inversion does not necessarily increase with the bare surface charge density of the interface and that even this concept of surface charge density may become meaningless in some biological ion channels. Thus, any theory based on electrostatic correlations or chemical binding should explicitly take into account the particular structure of the charged interface.

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I. INTRODUCTION

Among the rich and complex variety of physicochemical phenomena that can be found in biological media, the exchange of neutral and charged solutes across the cell membrane at different regulated rates is of particular importance [1]. The discrimination between charged solutes, known as ion selectivity, is a specialized physiological function exerted by some nanometer-sized pores known as ion channels that are ubiquitous in living organisms. Recently, we reported that the well-known moderate cationic selectivity found for the OmpF channel (a general diffusion porin located in the outer membrane of *E. coli*) in salts of monovalent ions [2–6] turns into anionic selectivity in concentrated solutions of barium, calcium, nickel, and magnesium chlorides [7]. Here we advance the experimental study aiming to elucidate if this inversion of selectivity (from cationic to anionic) is based on an effective charge inversion of the system. This phenomenon, known also as overcharging or charge reversal, occurs when interfacial charges attract counterions in excess of their own nominal charge [8–11]. Furthermore, we critically discuss whether existing theories of charge inversion can successfully be applied to the interface between the protein and the solution of a nanometer-sized biological channel.

Charge inversion was first studied systematically by Troelstra [11] and Bungenberg de Jong [12] more than 50 years ago, and it has been later reported in such diverse systems like lipid vesicles, colloids, Langmuir monolayers, nanofiltration membranes, flexible polyelectrolytes, and other synthetic nanodevices that are in contact with an aqueous solution containing multivalent ions [11–15]. The interpretation of such findings is still a matter of intense debate between different ways of tackling the problem.

Traditionally, charge inversion has been explained in terms of specific chemical interactions between ions and charged interfaces (by invoking hydrogen bonding, hydrophobic bonding, complex formation, or other solvent structure-mediated interactions) [10,11,16,17]. In this context, *specific* is the opposite of *generic* (long-range dispersion and electric interactions), and it implies that ions of the same valence could behave differently [16]. Thus, in a particular system meeting certain conditions about the charge stoichiometry and the relative separation between interacting charges [10], the phenomenological binding constant would summarize many different effects coming from the structure of the charged surface and its chemical composition, the ionic species in the solution and the counterion radii, hydrophobic interactions, etc. [18]. That empirical vision has been questioned because of its poor predictive power [19]. Actually, it involves many parameters that are often difficult to anticipate and can only be known from the fittings of the experimental data.

Alternative approaches emphasize that the conventional theoretical descriptions of electric double layers in charged interfaces (mean-field theories based on Poisson-Boltzmann equation) cannot explain the excess of counterions near a charged surface because they ignore ion-ion correlations [12,20]. The concept of *specific* binding is replaced by a universal *generic* mechanism for charge inversion based on the spatial correlations between the multivalent counterions in solution [10,21] that provide the loss of entropy needed for overcharging. In this framework the structure of the charged surface is secondary (note that correlation theories also lead to ion specificities caused by excluded volumes), so that essential qualitative features of many interfacial systems could be anticipated using idealized models like uniformly charged planes, spheres or cylinders, ions as charged hard spheres or the solvent as a dielectric continuum [8,12]. The lack of structural details is counterbalanced by the apparent conceptual simplicity and mathematical straightforwardness of strong correlation theories although in practice, such minimalism hinders a quantitative comparison with particular experiments [19]. The significance of correlation theories is a matter of controversy. On one hand it has been claimed that

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there are no alternative correlation-independent mechanisms leading to charge inversion and that correlations may appear in a large variety of ways including some usually unnoticed [12]. On the other hand, in a recent publication dealing with the quest for ion-ion correlations in systems conventionally described by specific chemical adsorption, it is suggested that although ion-ion correlations are theoretically well established, clear experimental evidence of them is virtually nonexistent [17].

The coexistence of such contrasting ideas opens a series of questions that we will address here on the basis of selectivity measurements in the OmpF channel: (i) unequivocal experimental determination of charge inversion: is there a unambiguous connection between the inversion of charge and the change of sign of a given measured quantity in all the charged interfaces explored?. (ii) Applying model systems to experiments: Is the "bare charge" of the interface-a key parameter in all theories of charge inversion-a meaningful quantity no matter the system considered? (iii) Universality of charge inversion mechanisms: Are ion-correlation and chemical-binding theories mutually excluding each other, or may there be a system where charge inversion admits both explanations?; (iv) Predictive power of current theories: both groups of theories agree in a prediction that the intensity or the probability of charge inversion increases with the surface charge density of the interface. Is this consistent with all experiments?

II. EXPERIMENTAL METHODS

Ion selectivity measurements have been performed on protein channels reconstituted on neutral lipid bilayer membranes. Wild-type OmpF isolated and purified from an *escherichia coli* culture was kindly provided by Mathias Winterhalter (Jacobs University, Germany). Bilayer lipidic membranes were formed from two monolayers prepared from 1% solution of diphytanoylphosphatidylcholine (DPhPC) (Avanti Polar Lipids, Inc.) in pentane (Baker) on 70–90-μm-diameter orifices in the 12-μm-thick Teflon partition that separated two chambers [7]. The orifices were pretreated with 1% solution of hexadecane in pentane. The total capacitance depended on the actual location of the orifice in the film but it was always around 70–130 pF. pH=6 was kept constant by 5 mM HEPES buffer. All measurements were performed at room temperature $(23.0 \pm 1.5)\,^{\circ}$C. Single-channel insertion was achieved by adding 0.1–0.3 μl of a 1 μg/ml solution of OmpF in the buffer that contained 1 M KCl and 1% (v/v) of Octyl POE (Alexis, Switzerland) to 2 ml aqueous phase at the cis side of the membrane only while stirring. The voltage, V, was applied via Ag/AgCl electrodes in 2 M KCl, 1.5% agarose bridges assembled within standard 200 μl pipette tips. Electric potential V is defined as positive when it is greater at the *cis* side of the membrane cell. An Axopatch 200B amplifier (Molecular Devices, Sunnyvale, CA) in the voltage-clamp mode was used. The membrane chamber and the headstage were isolated from external noise sources with a double μ-metal screen (Amuneal Manufacturing Corp., Philadelphia, PA).

The reversal potential, E_{rev}, was obtained as follows. First, a lipid membrane was formed at a given salt concentration gradient. Second, a single OmpF channel was inserted at zero potential and the channel conductance was checked by applying +50 mV and then switching potential polarity. Third, the ionic current through the channel was manually set to zero by adjusting the applied potential. The potential needed to achieve zero current was then corrected by the liquid junction potentials of the electrode salt bridges [7]. Each point was measured for at least three different channels in three different experiments to assure reproducibility and to estimate the standard deviation.

TABLE I. Ion selectivity of OmpF-WT in several salts (1/0.1 M). Measured reversal potential (mV).

KCl	NaCl	$CaCl_2$	$MgCl_2$	$LaCl_3$
-25.4 ± 0.8	-18.7 ± 1.1	22.1 ± 0.7	28.5 ± 0.8	42.8 ± 1.2

III. SELECTIVITY INVERSION BY MULTIVALENT IONS

Our study of the channel overcharging induced by multivalent ions is made on the basis of several series of selectivity experiments performed in the OmpF channel [22,23] reconstituted on planar phospholipid membranes. Recently [7], we reported preliminary measurements of OmpF selectivity in salts of monovalent and divalent cations. Ion selectivity was quantified, as usual, by measuring the reversal potential, E_{rev}, defined as the applied potential across the channel needed to get zero electric current when there is a concentration gradient between both sides of the channel. It was obtained that the sign of E_{rev} for chloride salts of divalent cations is opposite to that found for monovalent salts [7]. Later, we have extended the study to trivalent cations and checked that La^{3+} cations also yield selectivity inversion. Table I shows a summary of these results.

In the literature of ion channel biophysics, the selectivity experiments are customarily interpreted in terms of an effective channel charge. This concept refers to the charge that gives rise to the electric field actually felt by the ions permeating through the channel. This view could be misleading if one considers that effective charge refers not only to the channel charges (as we do here) but also to the overall solution flowing across it. Evidently, this is not the case because every macroscopic object must be neutral as a whole. In this sense, ions and channels are inseparable. The ionizable residues of the channel demand a countercharged ionic atmosphere to preserve macroscopic electroneutrality.

Thus, the effective negative charge resulting from the channel ionizable residues has been reported to be on the basis of the observed channel preference for monovalent cations over monovalent anions at neutral pH [4,5,24,25]. Following an intuitive reasoning one could speculate that the anion selectivity found for multivalent cations in Table I could come from an effective positive charge in the channel. Looking for solid arguments beyond intuition we study here how charge modifications (site-directed mutagenesis) influence the measured channel selectivity. The choice of the mutants is based on the results of some computational simulations [5] and experimental studies [3,6,26,27] that

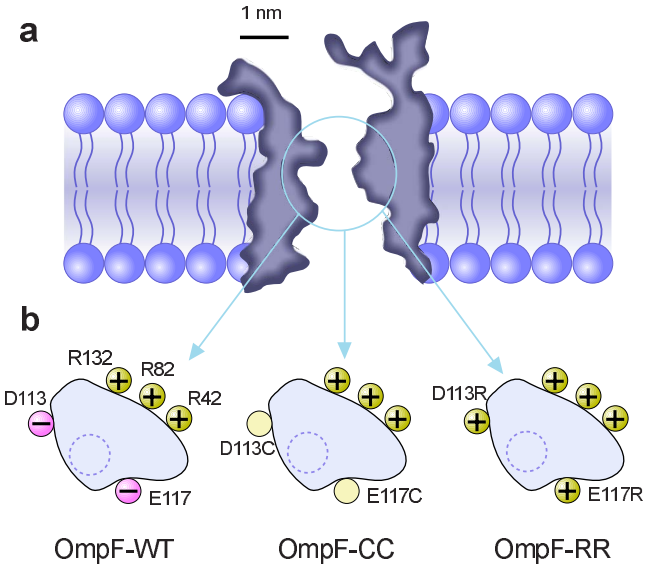

FIG. 1. (Color online) Sketch of the OmpF channel. (a) Longitudinal cross section. (b) Idealized cross section of the OmpF channel constriction for the wild-type (OmpF-WT) protein channel, and the mutants with residues D113 and E117 replaced with cysteines (OmpF-CC) or arginines (OmpF-RR). The dashed contour line represents the hypothetical binding site for a divalent cation based on the atomic structure of OmpF-WT in 1 M $MgCl_2$ [27].

demonstrate the crucial role exerted by some acidic residues lying on the narrowest part of the channel on its functional properties. More specifically, a recent x-ray crystal structure of OmpF in 1 M $MgCl_2$ revealed one Mg^{2+} ion bound at the channel constriction between two negatively charged residues: the aspartic acid D113 and the glutamic acid E117 [27]. Motivated by that study, we decided to investigate if the charge of D113 and E117 residues is indispensable to give rise to an inversion of selectivity. This was accomplished by measuring the reversal potential of the original (OmpF-WT) channel, and two mutants in which the above residues had been replaced either by two neutral cysteines (OmpF-CC) or by two positively charged arginines (OmpF-RR). Experimental characterization of these mutants showed that the size of the pore constriction is not significantly changed after chemical modification [26]. The dimensions of the central channel constriction are always large enough to discard steric or entropic effects as contributors to the measured selectivity. Figure 1 shows a sketch of the cross section of the OmpF channel constriction in the three cases mentioned. Control experiments of selectivity in monovalent KCl solutions were also carried out. Table II shows the reversal potential measurements in tenfold concentration gradients (1 M cis/0.1 M trans) at pH 6.

In a scenario ruled only by Coulombic interactions between charged residues and multivalent cations the elimination of two crucial negative charges should have a dramatic impact. But intriguingly, the substitution of the two negative residues D113 and E117 by two neutral ones (see OmpF-CC) does not have such critical effect. The cationic selectivity in salts of KCl is maintained and the selectivity inversion produced by Ca^{2+} ions in OmpF-WT is not removed. In fact, the anionic selectivity of OmpF-CC is even 50% higher than

TABLE II. Ion selectivity of OmpF (WT and mutants) in KCl and $CaCl_2$.

		E_{rev} (mV)	E_{rev} (mV)	Selectivity
OmpF channel	Δq [a]	1/0.1 M KCl	1/0.1 M $CaCl_2$	Inversion
WT	0	-25.4 ± 0.8	22.1 ± 0.7	Yes
CC	+2	-23.8 ± 0.8	30.1 ± 1.1	Yes
RR	+4	31.9 ± 1.0	35.4 ± 1.7	No

[a] Δq: effective charge compared to WT OmpF.

OmpF-WT. This can be interpreted in two ways: (a) the mutated residues do not play a relevant role in selectivity inversion (i.e., other negatively charged sites come into play); (b) even though the short-range interaction of divalent cations with the channel takes place at the constriction, it is not the charge of those negative residues but other structural factors what matters for selectivity inversion. Interestingly, when the OmpF-CC mutant is chemically modified with two negatively charged MTS compounds to resemble the original two-acid configuration, the inversion of selectivity is lost (see CC-MTSES mutant in Ref. [26]). This suggests that it is not the charge in the residues but the presence of particular functional groups in a precise arrangement what it is crucial in the interaction of multivalent cations with the channel. Indeed, the CC-MTSES mutant displays only 10% of the OmpF-WT conductance in salts of $CaCl_2$ despite having the same effective charge and a similar pore diameter [26].

The substitution of D113 and E117 by two arginines (OmpF-RR) makes the channel anion selective both in salts of monovalent cations (KCl) and divalent cations ($CaCl_2$). Therefore, we cannot speak of selectivity inversion in this case. These results are consistent with an anionic selectivity stemming from an effective positive charge of the channel. The slight difference ($\sim 10\%$) between reversal potential measured in KCl and $CaCl_2$ does not allow a trivial explanation because of the large diffusional contribution of $CaCl_2$ to E_{rev} in comparison with KCl. This point has been discussed elsewhere [7]. As in the CC-MTSES mutant, the fact that mutations in the constriction eliminate the selectivity inversion by calcium cations is an indication that the origin of charge inversion must be searched mainly in this narrow part of the channel rather than near other negative residues located either at the channel entrance or at the exit.

IV. SELECTIVITY INVERSION AND CHARGE INVERSION

The results presented hitherto suggest that there is no direct connection between the effective channel charge and the measured ionic selectivity. Thus, the rationalization of the channel selectivity inversion in terms of a surface where interfacial charges attract multivalent counterions in excess of their own nominal charge deserves further analysis. Does selectivity inversion necessarily mean charge inversion and vice versa? This question lies on the basis of the uncertainty about charge inversion since electrical charge is not directly measurable in most cases and charge inversion is discussed

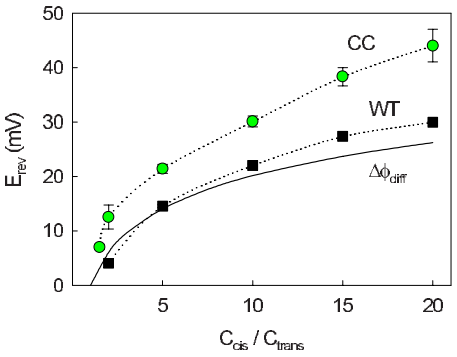

FIG. 2. (Color online) Reversal potential measured in $CaCl_2$ for OmpF-WT (squares) and OmpF-CC (circles) at pH 6. C_{trans} =0.1 M, and C_{cis} varies from 0.2 up to 2 M. Each point was measured for at least three different channels in three different experiments. The solid line denotes the bulk diffusion potential as explained in the text.

in terms of a certain physical quantity regulated by the effective electrical charge (electric potential, streaming potential, force, capacitance, ion selectivity, etc.) [11,12,19].

Ionic selectivity, i.e., the channel ability to discriminate between ions, is a property of the system that inevitably includes both the channel and the electrolyte solution flowing through it. Consequently, several factors contribute mainly to the observed ion channel selectivity: the diffusional effects coming from the differences in ion mobilities and the electrostatic exclusion due to the interaction between permeating ions and channel ionizable residues. Other factors such as entropic effects related to the partial rejection of ions because of their size, short-range nonelectrostatic interactions, and hydrodynamic hindrance might play a role in certain specific cases [7].

Figure 2 shows the reversal potential measurements in salts of $CaCl_2$ for the two channels that display selectivity inversion: OmpF-WT (squares) and OmpF-CC (circles). Each series of experiments was made at pH 6 by keeping constant the salt concentration on trans side at C_{trans} =0.1 M and varying the salt concentration on cis side (the side of the protein addition) from C_{cis}=0.2 M up to 2 M. The measured E_{rev} was positive over the whole range of concentration gradients, which means that transport of anions is favored over cations. In order to clarify whether the measured zero current potential is associated with a property of the channel itself or it is a feature of the electrolyte, we have also plotted the theoretical estimation of the diffusion potential of the $CaCl_2$ electrolyte, according to Planck's expression for a $z_+:z_-$ binary electrolyte (z_i include the sign) [7],

$$\Delta\phi_{\text{diff}} = \left(\frac{k_B T}{e}\right)\frac{D_- - D_+}{z_+ D_+ - z_- D_-}\ln\frac{C_{cis}}{C_{tr}} \qquad (1)$$

where k_B and T have their usual meaning of Boltzmann's constant and absolute temperature and e is the elementary charge. D_i denote the ionic diffusion coefficients (in the plot we use infinite dilution values for D_i).

TABLE III. Selectivity inversion of OmpF-WT and OmpF-CC.

Salt solutions 1 M/0.1 M	Bulk diffusion potential (mV)	OmpF-WT E_{rev} (mV)	OmpF-CC E_{rev} (mV)
$BaCl_2$	19.0	22.0 ± 0.3	29.7 ± 1.9
$CaCl_2$	20.1	24.5 ± 0.7	32.9 ± 1.1
$NiCl_2$	23.7	28.4 ± 0.5	42.9 ± 1.0
$MgCl_2$	22.4	28.5 ± 0.8	44.0 ± 2.3
$LaCl_3$	21.3	42.8 ± 1.2	48.7 ± 0.5

Note that under physiological conditions (moderate gradients of KCl solutions buffered at neutral pH) diffusion potentials are negligible because K^+ and Cl^- have almost equal bulk mobilities. This allows one to reduce the selectivity to electrostatic exclusion only and to interpret it exclusively in terms of the effective channel charge. But in experiments with other electrolytes the description of selectivity just in terms of ion accumulation/depletion could be an oversimplification of the problem

The diffusion potential shown in Fig. 2 (solid line) would be the measured reversal potential in a neutral ideal channel devoid of any electrostatic interaction and just filled with a $CaCl_2$ solution. One could argue that diffusion coefficients of ions inside the channel could differ from their tabulated free-solution values. Indeed, previous studies indicate that the cation/anion mobility ratio inside the channel could be slightly reduced in respect to its bulk value [6,7]. In such case, the actual diffusion potential would be greater than that predicted by Eq. (1). Since this effect is difficult to quantify exactly, Eq. (1) can be considered as a lower limit of the diffusion potential. Hence, Fig. 2 indicates that the measured reversal potential for OmpF-WT in $CaCl_2$ is mostly, if not wholly, due to the different mobilities of anions and cations. This means that divalent cations compensate in some way the negative charge arising from the channel ionizable residues, almost cancelling the electrostatic exclusion of cations. Therefore, such anionic selectivity cannot be unequivocally linked to a effective positive charge in the channel.

Table III shows that this is not a peculiarity of $CaCl_2$, but it is also exhibited by other salts of divalent cations ($BaCl_2$, $MgCl_2$, and $NiCl_2$). Note that in all cases the bulk diffusion potential (calculated according to Eq. (1) for a 1/0.1 M gradient) is at least 80% of the measured E_{rev} for OmpF-WT. In contrast, the reversal potentials for OmpF-CC measured under the same conditions indicate both quantitatively and qualitatively (Fig. 2 and Table III) that, in addition to diffusional effects, supplementary sources of anionic selectivity must be present and the inversion of the effective charge seems possible in the OmpF-CC mutant.

With regard to $LaCl_3$, the inversion of the channel charge seems evident both in OmpF-CC and OmpF-WT since the measured E_{rev} is twice the corresponding bulk diffusion potential. The elimination of negative charges (see OmpF-CC) does not remove but enhances the interaction between trivalent cations and the channel, pointing again to structural factors. Note also that the E_{rev} measurements reported in Tables I–III prove very clearly that ion selectivity is not an intrinsic

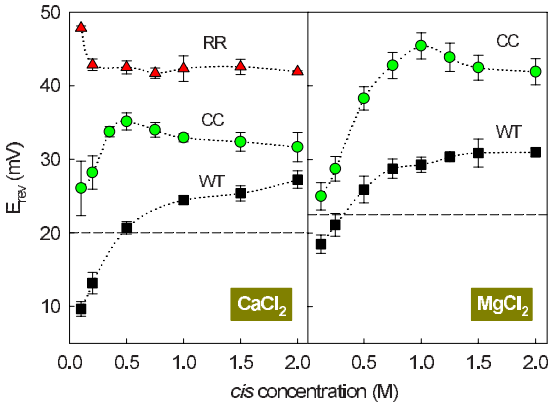

FIG. 3. (Color online) Reversal potential measured for OmpF-WT (squares) and mutants OmpF-RR (triangles) and OmpF-CC (circles) at pH 6 in $CaCl_2$ (left) and $MgCl_2$ (right). $C_{cis}/C_{trans}=10$ and C_{cis} varies from 0.2 up to 2 M. Each point was measured for at least three different channels in three different experiments. The dashed lines denote the corresponding bulk diffusion potentials in $CaCl_2$ and $MgCl_2$.

property of the channel but necessarily includes the electrolyte flowing through it. The same channel can be selective to cations, neutral, or selective to anions without requiring pH titration of the channel charges.

To further investigate the nature of the anionic selectivity found in OmpF-WT and OmpF-CC mutant we did additional experiments with the mutant OmpF-RR, an anion selective channel both in KCl and $CaCl_2$ (see Table II). Figure 3 shows several series of measurements of the reversal potential at tenfold *cis/trans* concentration ratio but different absolute concentrations of $CaCl_2$ (left panel) and $MgCl_2$ (right panel). By keeping invariable the concentration ratio, the diffusional contribution should be approximately constant [see Eq. (1)] and we could study, at least qualitatively, how other factors contribute to the overall selectivity. The dashed lines denote the corresponding bulk diffusion potentials in $CaCl_2$ and $MgCl_2$. For the three channels E_{rev} depends not only on the concentration ratio but also on the absolute concentration though in a different manner.

The measurements for the OmpF-RR mutant show the expected behavior of an anion selective channel ruled by the electrostatic exclusion of cations characteristic of a channel with effective positive charge. As salt concentration increases the screening of channel fixed charges is more effective and selectivity decreases [4].

OmpF-WT data show no evidence of such anionic exclusion. The low-concentration limit of OmpF-WT data in Fig. 3 is below the diffusion potential, which means that in such limit the channel retains part of its intrinsic negative fixed charge (note that the contribution of the cationic electrostatic exclusion to E_{rev} is negative, whereas bulk diffusion potentials in $CaCl_2$ and $MgCl_2$ are positive). Increasing the salt concentration produces a rise in the reversal potential, suggesting that the interaction of divalent cations gradually balances the negative charge of the channel. Despite the fact that the measured reversal potentials for OmpF-WT are very close or even exceed the calculated diffusion potentials (for a ratio $C_{cis}/C_{trans}=10$ of $CaCl_2$ $\Delta\phi_{diff}=20.2$ mV) we cannot assure the existence of a charge inversion phenomenon in the OmpF-WT channel. First because as mentioned above, the actual diffusion potential can be slightly higher than that predicted by Eq. (1). Second, no typical anionic screening such as that observed for the OmpF-RR mutant is displayed in this case. Thus, the available data for OmpF-WT are more consistent with an almost neutral channel where electrostatic exclusion (accumulation) of cations (anions) is a secondary source of selectivity and diffusional effects are predominant both qualitatively (shape of the curve in Fig. 2) and quantitatively. In contrast to what would seem intuitive, selectivity inversion does not necessarily mean charge inversion.

Let us analyze the nonmonotonic concentration dependence of E_{rev} displayed by OmpF-CC. Figure 3 shows that at low concentration the OmpF-CC mutant behaves qualitatively like the OmpF-WT but with a crucial quantitative difference: E_{rev} measurements in OmpF-CC are always higher than the calculated diffusion potential both in $CaCl_2$ and $MgCl_2$ solutions. This means that even at low concentration the channel does exclude cations, in contrast to what happens in KCl solutions (see Table I). Since we see a gain of anionic selectivity with increasing concentration, we conclude that a concentration-dependent interaction of Ca^{2+} or Mg^{2+} ions with the protein is the mechanism responsible for selectivity inversion. Interestingly, reversal potential data for OmpF-CC attain a maximum and then slightly decrease with increasing concentration. In experiments with $CaCl_2$ for $C_{cis}>0.5$ M (or $C_{cis}>1$ M in $MgCl_2$), E_{rev} change with concentration follows the pattern of electrostatic screening as in the OmpF-RR mutant. This would be consistent with the existence of an effective positive charge (i.e., a charge inversion phenomenon) that is a supplementary source of ion selectivity in addition to diffusional effects. Note that in this concentration range E_{rev} exceeds the diffusion potential by almost a factor of two, so that the anionic selectivity points to a positive effective charge of the channel.

A tentative explanation for the observations in OmpF-CC mutant could be the following. In the low-concentration limit, the OmpF-CC mutant is not far from electrical neutrality (that is the reason why the measured reversal potential is similar to the calculated diffusion potential): in such a picture, divalent cations do not suffer from significant electrostatic exclusion and can enter easily in the channel and interact with the surface charges. This interaction has two main effects contributing to the overall selectivity in opposite directions. On the one side, the overaccumulation provides additional positive charge to the channel and hence produces a gain in anionic selectivity. But, on the other side, this gain in anionic selectivity hampers the entrance of new divalent cations into the channel since they are now electrostatically excluded. The result from this competition would be the nonmonotonic curve observed.

V. ION CORRELATIONS AND CHEMICAL BINDING

We have shown above that the selectivity inversion observed in OmpF-WT is not probably an evidence of charge inversion. What are the predictions of the theories of charge inversion? According to the models invoking correlations be-

tween counterions [12] the magnitude of the coupling is quantified by the plasma parameter Γ defined as: $\Gamma=(Z_+^2e^2/\varepsilon R k_B T)$ where $R=(Z_+e/\pi\sigma)^{1/2}$. For salts of divalent cations ($Z_+=2$), bulk water ($\varepsilon=78$), and an average value for OmpF "bare" surface charge density $\sigma=1$ e/nm^2 [4,24,25] we obtain a remarkably coupled system with $\Gamma\approx 4$. Moreover, if we take into account recent studies showing that dielectric properties of water inside the channel can differ dramatically from that in bulk water [28], the effective dielectric constant of water could be $\varepsilon\approx 30$ and hence $\Gamma\approx 10$. Alternatively, we can calculate the parameter $\zeta=(Z_+e/\pi\sigma\lambda_D)$ where λ_D is the Debye length for a 1 M solution of a salt of a divalent cation. Thus, we obtain $\zeta \gg 1$ for reasonable values of ε ($\zeta\approx 10$ for $\varepsilon=78$ and $\zeta\approx 30$ for $\varepsilon=30$). Both Γ and ζ indicate that divalent cations could reasonably form a strongly correlated liquid providing the favorable free energy required for charge inversion.

Alternatively, we can also consider a more "chemical" picture dominated by the binding between divalent cations and interfacial charges with an equilibrium constant K for the binding reaction. If only Coulombic interactions are involved, the binding constant can be computed following Bjerrum pairing theory of electrolytes [29]. According to this model, K depends essentially on the valence of the interfacial charges (again the "bare" surface charge density) and on structural characteristics (crystallographic radius of bound ions, Bjerrum length of the electrolyte). Bjerrum pairing requires that oppositely charges come close enough so that they attract more strongly than the disordering thermal fluctuations and binding occurs. According to Travesset and Vaknin [29] this typical distance D should be lower than a certain quantity $(l_B Z_+ Z_-/2)\approx l_B$, where $l_B\approx 7$ Å is the Bjerrum length. $Z_+=2$ and $Z_-=1$ is assumed to be the charge of the interfacial acidic molecule to which the counterion is bound. Having in mind that 7 Å is the diameter of the OmpF channel central constriction, we can assure that the distance between interfacial charges and permeating counterions is less than this value. This clearly suggests that the binding of divalent cations to the channel surface charges could also provide the favorable free energy required for charge inversion.

The two models give very similar predictions for OmpF-WT, OmpF-CC, and OmpF-RR because the difference in the "surface charge density" σ is minimal. However, our experiments reveal that the channel selectivity is completely different. Therefore, it becomes apparent that additional details of the multivalent cation interaction with the residues at the channel constriction should be incorporated in the model. In other words, the interpretation of such experiments in the light of current theories of charge inversion deserves a critical discussion on the meaning of the fundamental parameter of those models: the "bare charge" of the interface [11,12].

VI. APPLYING MODEL SYSTEMS TO EXPERIMENTS

The experiments with OmpF channels reported here make clear that the connection between selectivity and charge is not as direct as one might think. Thus, the inversion of selectivity found in salts of multivalent cations does not imply an overcharging of the channel fixed charges but can be caused by nonelectrostatic mechanisms.

We have shown how in some cases the effects of differences in ion mobilities could prevail over the effects of electrostatic exclusion. The two mechanisms are not independent since both are simultaneously ruled by local concentration of mobile ions [7]. But even if we are able to separate the exclusion and the diffusion contribution to reversal potential, many queries immediately arise. What is the effective charge of the channel responsible for electrostatic exclusion? Is it the effective charge on the pore inner wall the "bare charge" of the interface? Should we consider the whole channel to calculate it or should we restrict ourselves to the some small region where the x-ray crystal structure locates the divalent cations? [27] In the OmpF case, these questions make a lot of sense: previous studies indicate the ion transport is not controlled by certain charges in particular locations of the pore. Quite the opposite, ion selectivity is ruled by the collective action of a large number of ionizable residues [4,24,26].

The oversimplified picture of a homogeneous negatively charged surface easily accessible to multivalent cations has little meaning here: OmpF porin is an intricate amphoteric structure where positive and negative charges alternate along the pore inner surface yielding a positive effective charge. Only when buried residues are taken into account can one explain the observed channel preference for monovalent cations [25]. But those negative charges buried in the low dielectric protein environment are clearly inaccessible to multivalent cations. Moreover, even worse, if we trust the x-ray crystal structure and assume that the charge inversion takes place at the channel constriction, the effective charge in that small region (~ 1 nm^3) is also positive [4].

The conclusion is obvious: either we consider the whole channel or the small central constriction, the effective charge of the interface is positive, opposite to what would be needed for attracting cations. The reason why multivalent cations reverse the channel selectivity must inevitably include other factors in addition to interfacial charges. The concept of *bare* charge or surface charge density usually considered the pivotal magnitude in theories of charge inversion appears to be meaningless here.

The experiments presented here add support to this statement. The large differences in selectivity (and selectivity inversion by multivalent cations) found among OmpF-WT, OmpF-CC and OmpF-RR as well as the binding site for Mg^{2+} revealed by a recent OmpF crystal structure suggest that charge inversion occurs near residues D113 and E117 although the charge of these sites is not essential. In this sense it is important to note that when residues D113 and E117 are replaced by neutral cysteines in the OmpF-CC mutant, the structure of the inner hydrocarbon chain (where the divalent cation is shown to be placed [27]) is preserved. This is an indication that the specific coordination of water molecules around the Mg^{2+} ion shown in the crystallographic structure of OmpF-WT can be maintained as well in OmpF-CC. If this is true, it is hard to think of a distribution of counterions in such a small length scale (below 1 nm). This is a major, evident challenge to current theories of charge inversion.

So we find ourselves bound to admit that when we talk here about charge inversion, it does not necessarily mean

overcharging of interfacial charges by multivalent counterions. It is safer to say that the observed channel preference for anions (what is meant by anionic selectivity) is not a pure surface effect based on the charge. Rather, it is likely a combined effect of the long-range Coulombic interactions between protein and mobile charges and the short-range interaction involving particular functional groups in a precise arrangement. The existence of a common underlying universal mechanism for charge inversion in such diverse interfacial systems like colloids, microchannels, nanopores, macroions, biological channels, etc. is difficult to reconcile with the number of particular details needed to rationalize our observations in OmpF.

VII. CONCLUSIONS

The major findings of the present paper can be then summarized as follows:

(i) selectivity inversion in a large biological ion channel does not imply necessarily that the channel effective charge reverses its sign. The influence of differences in mobile ion diffusivities should be taken into account.

(ii) A detailed knowledge of the charged interface structure is mandatory to assure that charge inversion takes place. We show that a meticulous scrutiny of a large number of experiments involving channels with selected mutated residues and several electrolytes is needed to infer charge inversion from selectivity inversion. This raises the question of whether there is a common underlying mechanism in all observations reported under the name of "charge inversion."

(iii) A reduction in the channel effective charge causes an increase in selectivity inversion, contrary to what current theories predict for a homogeneously charged interface. We note that in the OmpF channel, the surface charge density in the pore wall is a meaningless concept.

(iv) The generality and predictive power of current theories of charge inversion invoking model systems could be challenged by their lack of essential structural details needed to analyze a particular system. In the present case, besides the channel charges and the counterion valence, entropic considerations about the precise configurational channel arrangement in the presence of divalent cations seem decisive.

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