Role of Electroosmosis in the Permeation of Neutral Molecules: CymA and Cyclodextrin as an Example

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ABSTRACT To quantify the flow of small uncharged molecules into and across nanopores, one often uses ion currents. The respective ion-current fluctuations caused by the presence of the analyte make it possible to draw some conclusions about the direction and magnitude of the analyte flow. However, often this flow appears to be asymmetric with respect to the applied voltage. As a possible reason for this asymmetry, we identified the electroosmotic flow (EOF), which is the water transport associated with ions driven by the external transmembrane voltage. As an example, we quantify the contribution of the EOF through a nanopore by investigating the permeation of α-cyclodextrin through CymA, a cyclodextrin-specific channel from Klebsiella oxytoca. To understand the results from electrophysiology on a molecular level, all-atom molecular dynamics simulations are used to detail the effect of the EOF on substrate entry to and exit from a CymA channel in which the N-terminus has been deleted. The combined experimental and computational results strongly suggest that one needs to account for the significant contribution of the EOF when analyzing the penetration of cyclodextrins through the CymA pore. This example study at the same time points to the more general finding that the EOF needs to be considered in translocation studies of neutral molecules and, at least in many cases, should be able to help in discriminating between translocation and binding events.

INTRODUCTION

In single-channel electrophysiology experiments, the permeation of molecules across pores depends often on the magnitude and direction of the applied voltage (1). For charged molecules, the electrophoretic force experienced depends obviously on the polarity of the applied voltage, leading to an asymmetry in kinetics (2,3). However, in the case of uncharged molecules, the reason for this asymmetry remains unclear (4–6). In a few cases, such asymmetry is claimed to be due to electroosmotic flow (EOF), that is, the net flow of water molecules dragged along by ions in the presence of an electric field (1,7–10). This effect has been known since the early days of channel characterization, but due to the complexity of ion interaction, it is impossible to predict in a quantitative manner by a simple equation. For example, one of the first studies in a gramicidin channel elucidated the number of water molecules and the streaming potential at different osmolar conditions (11,12). Since then, numerous studies have been performed, for example, using molecular dynamics (MD) simulations in bulk (13) and artificial/biological channels (14–17). Moreover, the transport of polymers (18) and DNA (19) through artificial nanopores, which might be highly charged, has been detailed in MD simulations (20,21). With respect to biological nanopores, however, the relevance of the EOF in polymer transport is still under debate (22,23), with a few exceptions. In a seminal article, Gu and co-workers (1) lodged β-cyclodextrin (β-CD) in the channel lumen of an α-hemolysin pore in a noncovalent manner. They performed site-directed mutagenesis studies to achieve prolonged residence times of β-CD in the channel by altering the selectivity of the channel and thereby the water flow. More recent studies have shown the voltage dependence of the EOF in α-hemolysin using the two different salts LiCl and KCl, and a theoretical model that considers the pore selectivity and solvation of ions has been proposed (7). A detailed understanding of the EOF influence on the translocation of neutral molecules is of general interest for the nanopore community. Applying external transmembrane potentials to indirectly measure the passage of uncharged molecules reveals voltage-dependent rates of channel blockage (24–28). From modifications of substrate residence times in the channel, we can draw conclusions as to the mode of transport, i.e., translocation or binding. Among others, a potential area for application is in the field of finding new antibiotics. A current identified bottleneck is the low permeability in Gram-negative bacteria. Having a method that allows screening for molecules with rapid channel permeation would make it possible to find scaffolds to design antibiotics with improved permeability. Such
molecules would be active at lower concentrations and thus would reduce the toxic side effects.

Here, we study the contribution of the EOF to the permeation of a neutral molecule, α-CD, through the CD-specific channel CymA from Klebsiella oxytoca (29–31). CymA is a monomeric outer-membrane protein with a diameter of 12–14 Å that mediates the passive uptake of bulky CD molecules with molecular mass >600 Da. It folds as a 14-stranded β-barrel with an N-terminal overhang toward the periplasmic side of the channel. Inspection of the structure reveals, in contrast to other porins, an almost cylindrical pore with only the N-terminal loop as constriction. Removing the first 15 residues of the N-terminus by site-directed mutagenesis (shown in Fig. 1A) leads, in lipid bilayer experiments, to an electrically silent pore of ~1.2 nS conductance in 1 M KCl (10 mM MES) at pH 6 (32). Based on the simplicity of the channel, we performed the experiments on the deletion mutant of CymA, denoted as ΔCymA, to elucidate the significance of EOF.

The magnitude and direction of the EOF depends on various factors, including diameter and selectivity of the channel, nature of electrolytes, applied voltage, temperature, pH, etc. Here, we have employed different electrolyte solutions, including KCl, NaCl, and MgCl2, to investigate the influence of these mobile charge carriers on channel electrostatic potential screening, thereby manipulating the selectivity of the channel. Furthermore, we considered the polarity of the applied voltage to elaborate its effect on the direction of the EOF. We have carried out bilayer measurements with complementary free-energy calculations and applied-field MD simulations to detail the transport of various ionic species across the ΔCymA pore. Selectivity measurements carried out for the monovalent cation-containing salts KCl and NaCl indicate the preference of cations over anions. Interestingly, we were able to achieve an inversion in the channel electrostatic potential, mediated by divalent magnesium ions reversing the cationic to anionic selectivity, without resorting to any chemical modification of the channel. We performed substrate interaction studies where we report a voltage-dependent change in the association rate (k_{assoc}) and residence time (τ) deduced from the voltage and effector concentration-dependent single-channel gating (33,34) for the neutral α-CD entering the ΔCymA channel. Moreover, we have quantified the magnitude and direction of the net water flux (EOF) using applied-field MD simulations. Based on the calculated EOF for the three studied ionic solutions, we were able not only to show the existence of the EOF but also to correlate the voltage-dependent changes in association rate and residence time of α-CD across the ΔCymA channel with the net water flux. To further elucidate the significance of the EOF at the atomic level, unbiased, applied-field, and steered MD simulations were carried out on the cocrystallized structure of α-CD bound to the extracellular side, the so-called entry site of the CymA channel. From unbiased and applied-field simulations, we could clearly demonstrate the water-mediated destabilization of a substrate from the entry site. Moreover, the influence of the EOF was also reflected in the force obtained from the steered MD simulations carried out at different applied voltages.

To utilize the EOF as a potential tool in discriminating between translocation and binding, the control experiments were carried out using probable nontranslocating molecules, β- and γ-CD. From these results, we were able to demonstrate the need for the contribution of EOF while analyzing the rate kinetics of uncharged molecules obtained from single-channel experiments. As a final remark, we also compared the strength of the osmotic flow with that of EOF at given experimental conditions by performing hydrostatic pressure-driven MD simulations. All together, we show here a significant example of how detailed atomic-level insight obtained from MD simulations on EOF can help us to understand the electrophysiology results obtained at the macroscopic level.

**MATERIALS AND METHODS**

**Construction and purification of ΔCymA**

ΔCymA was constructed, expressed, and purified as described previously (31). In short, the oligonucleotide primers FP (5′-GAAAGTTTT TTTCGTTTGGTGGCCAT-3′) and RP (5′-TGCAATGATGTAC GGCCGCTGTGA-3′) were used to amplify pCymA using the Phusion DNA polymerase from Thermo Fisher Scientific (Waltham, MA). Subsequently, the polymerase chain reaction product was subjected to DpnI digestion and T4 polynucleotide kinase phosphorylation. The phosphorylated product was subjected to ligation using T4 DNA ligase (Thermo Fisher Scientific) and electroporated into DH5α cells. To purify ΔCymA, cells were grown at 37°C overnight in lysogeny broth medium, harvested by centrifugation and homogenized in 50 mM potassium phosphate with protease inhibitor (1 mM phenylmethylsulfonyl fluoride) at pH 7.5 (buffer A). Cells were disrupted by passing through a French press three to five times at 16,000 psi followed by centrifugation at 6000 × g for 1 h to separate them from the intact cells. Cell envelopes were obtained by ultracentrifugation at 100,000 × g for 90 min. The inner membrane components were solubilized in 0.5% sarcosyl containing buffer A, stirred for 1 h at room temperature, and centrifuged at 100,000 × g for 90 min. This procedure was repeated one time and the pellet thus obtained was resuspended in 100 mg/mL lysozyme and 3 mM NaNO3 containing buffer A, with overnight stirring at ≥7°C, which was further subjected to ultracentrifugation at 100,000 × g for 90 min. Then, 5 mM EDTA and 5% octyl-polyoxyethylene (OPOE; Bachem Biochemica, Heidelberg, Germany) in buffer A was employed to solubilize the outer membrane and centrifugation was performed. The supernatant thus obtained was dialyzed against 0.5% octyl-polyoxyethylene (OPOE; Bachem Biochemica, Heidelberg, Germany) in buffer A and electroporated into DH5α cells. To purify ΔCymA, cells were grown at 37°C overnight in lysogeny broth medium, harvested by centrifugation and homogenized in 50 mM potassium phosphate with protease inhibitor (1 mM phenylmethylsulfonyl fluoride) at pH 7.5 (buffer A). Cells were disrupted by passing through a French press three to five times at 16,000 psi followed by centrifugation at 6000 × g for 1 h to separate them from the intact cells. Cell envelopes were obtained by ultracentrifugation at 100,000 × g for 90 min. The inner membrane components were solubilized in 0.5% sarcosyl containing buffer A, stirred for 1 h at room temperature, and centrifuged at 100,000 × g for 90 min. This procedure was repeated one time and the pellet thus obtained was resuspended in 100 mg/mL lysozyme and 3 mM NaNO3 containing buffer A, with overnight stirring at ≥7°C, which was further subjected to ultracentrifugation at 100,000 × g for 90 min. Then, 5 mM EDTA and 5% octyl-polyoxyethylene (OPOE; Bachem Biochemica, Heidelberg, Germany) in buffer A was employed to solubilize the outer membrane and centrifugation was performed. The supernatant thus obtained was dialyzed against 0.5% OPOE in 10 mM potassium phosphate, pH 7.5 (buffer B). As a final step, the final extract thus obtained was loaded onto a Mono Q-HR 5/5 column and eluted in buffer B containing 1 M NaCl on a linear gradient scale. The fraction containing ΔCymA was concentrated and stored at −80°C.

**Single-channel electrophysiology**

Employing the Montal and Muller technique, solvent-free membranes were produced as described previously (35). In short, a 25-μm-thick Teflon film with an aperture of ~50–100 μm was sandwiched between the two chambers of a Teflon cuvette. Then, 1% hexadecane in n-hexane was used to impregnate the aperture to confer a hydrophobic nature. Unless stated
otherwise, the two chambers were filled with electrolyte solution buffered with 10 mM MES at pH 6. The aqueous phase was buffered with 10 mM MES at pH 6. A solution of 5 mg/mL diphtyrylphosphatidylcholine in n-pentane was used to form the membrane. Furthermore, the electric current was measured using a pair of Ag/AgCl electrodes (World Precision Instruments, Sarasota, FL), where one was connected to the cis side of the membrane (ground) and the other was connected to the headstage of the Axopatch 200B amplifier (Axon Instruments, Foster City, CA). A detergent solution containing pure protein was added to the cis side of the membrane and the current measurements were done in the voltage-clamp mode of the Axopatch 200B amplifier and digitized using the Axon Digidata 1440 digitizer while the data acquisition was performed using Clampex software (Axon Instruments). Using a low-pass Bessel filter with a sampling frequency of 50 kHz, the traces thus obtained were filtered at 10 kHz. The data were analyzed by the Clampfit software. To determine the kinetics, a single ΔCymA channel was reconstituted and 10 mM α-CD was added to the extracellular side. A potential was applied and measured between the periplasmic side (cis, ground, side of protein addition) and the extracellular side of the channel. To evaluate the on rate, k_on, and residence time, τ, a single-channel analysis was performed (27). The on rate, k_on, is given by the number of binding events divided by concentration, whereas the residence time, τ, is given by the exponential fit of the dwell-time histogram. To determine the ion selectivity of the channel in various environments, 200–1500 ΔCymA channels were reconstituted in solvent containing membranes and zero-current potentials were recorded by establishing a salt concentration gradient across membranes, as described previously (36).

MD simulations

All simulations were performed using the GROMACS 4.6.5 package (37) together with the standard CHARMM36 force field (38). The short-range electrostatics and van der Waals interactions were calculated with a cutoff of 12 Å, whereas the long-range electrostatics was treated using the particle-mesh Ewald method with a grid size of 1 Å. Moreover, all bonds were constrained using the LINCS method to enable a time step of 2 fs. After the energy minimization, a constant temperature was maintained at 300 K using a Nosé-Hoover thermostat (39) and pressure was controlled using a Parrinello-Rahman barostat (40) at 1 bar. The simulation system consisted of the ΔCymA mutant porin without the N-terminus (residues 1–15) inserted into the 1-palmitoyl,2-oleoyl-sn-glycero-3-phosphocholine bilayer with 228 lipids. Moreover, the system was solvated using the TIP3P water model and subsequently three systems were constructed by adding 1 M KCl, 1 M NaCl, and 1 M MgCl₂, leading to a system size of ~90,000 atoms each. The applied field simulations (41–44) with different applied voltages between ~1 V and +1 V were performed for simulation times of up to 100 ns.

To get insight into the interactions of the various ion species with the pore, metadynamics simulations were carried out by enhancing the sampling of ionic configurations using the FEP protocol. All simulations, ions were placed near the mouth of the channel and a bias potential with a height of 1 kJ mol⁻¹ and a width of 0.1 Å was added every 4 ps to the distance of the collective variable. The collective variable was defined as the distance between the respective ion and the center of mass of the Cα atoms of the protein along the channel axis. The method of choice for performing the free-energy calculations was the well-tempered (45) and multiple-walker (46) algorithms implemented in PLUMED version 2.0.1 (47).

To investigate the effect of the EOF on a substrate directly, MD simulations of the α-CD at the entry site of the pore were performed in the absence and presence of 1 M KCl or 1 M MgCl₂. As the EOF was quite significant when α-CD was added at the extracellular side, we chose the entry site where α-CD was bound to the extracellular mouth in the crystal structure (PDB: 4D5B) as the starting structure. The ΔCymA mutant structure together with the α-CD at the entry side was inserted into a 1-palmitoyl,2-oleoyl-sn-glycero-3-phosphocholine lipid bilayer and the above-mentioned procedure for further system setup and equilibration was followed. In addition, the three glutamic acid residues (Glu80, Glu85, and Glu205) were kept in their protonated states to anchor the α-CD with high affinity (32). The simulations were carried out at three different applied voltages (−1, 0, and +1 V) for 50 ns in the absence and presence of the above-mentioned salts. Finally, steered molecular dynamics (SMD) simulations (48) were performed at the above-mentioned voltages to study the role of water in the dissociation of α-CD from the entry site in the presence of 1 M KCl or 1 M MgCl₂. In all simulations, the α-CD was pulled toward the bulk on the extracellular side at a constant velocity of 1 Å·ns⁻¹ and a spring constant of 100 kJ mol⁻¹ nm⁻² was used.

Concerning the calculations of the osmotic water flow through the ΔCymA channel, we followed a procedure detailed in the literature (49). The pressure difference on the two sides of the membrane was induced by enforcing an external force on all water molecules by carrying out SMD simulations. From the resulting trajectories, the number of water molecules entering and crossing the pore per second was determined as a function of the applied pressure difference. From the slope of this relation, one can deduce the hydraulic permeability coefficient, kₚ. Moreover, the osmotic permeability coefficient, pₒ, can be determined using the relation

\[ pₒ = \left(\frac{RT}{V_w}\right)kₚ \]

in which \( V_w \) denotes the molar volume of water (18 cm³/mol) (49).

RESULTS AND DISCUSSION

Structural characteristics of the ΔCymA channel

Biological nanopores contain a network of charged residues lining the interior of the channel, and these residues are mainly responsible for the ion selectivity of the pore (50,51). Depending on the density of charged residues, the electrostatic potential shows an asymmetry with respect to the channel axis (52). When an external electric field is applied, this asymmetry leads to a selective uptake of ions, resulting in cation or anion selectivity. As shown in Fig. 1 B, the CymA channel contains a dense network of negatively charged residues especially toward the extracellular side of the channel. In addition, more negatively than positively charged residues are present inside the channel lumen. This asymmetric charge distribution could make CymA a cation-selective pore. To get an initial insight into the interaction of different ion species with the ΔCymA channel, free-energy calculations were performed for four different ion types, i.e., K⁺, Na⁺, Mg²⁺, and Cl⁻ ions, within the channel. As can be seen in Fig. 1 C, Mg²⁺ ions experience a well depth of ~−21 kcal/mol, K⁺ ions of ~−14 kcal/mol, and Na⁺ ions of ~−9 kcal/mol, whereas chloride ions face a high energy barrier of ~14 kcal/mol in the constriction region. These simulations clearly suggest that the dense network of negatively charged residues inside the channel lumen makes the pore cation selective.

Ion transport through ΔCymA channel

Next, we studied experimentally and theoretically the ionic current through the ΔCymA to achieve an understanding of the architecture and electrostatics of the channel (41,43,53). We performed single-channel electrophysiology experiments where we reconstituted ΔCymA channels into a
diphytanoyl phosphatidylcholine membrane. We measured the ion current in three different salt solutions, i.e., 1 M KCl, NaCl, and MgCl$_2$, and compared it to the values obtained theoretically from applied-field simulations. The corresponding experimental and theoretical $I$-$V$ curves are depicted in Fig. 2, and they clearly show the voltage-dependent linear increase in the ionic current. The MD simulations agree very well with the experiments when comparing the conductance values in Fig. S1 in the Supporting Material. More interestingly, a slight asymmetry in conductance was observed for 1 M KCl in bilayer experiments with respect to the polarity of voltage applied: 1.2 nS at $+100$ mV and 1 nS at $-100$ mV. Such asymmetry was also observed for the other two investigated electrolytes in the bilayer experiments. The larger hydrodynamic radius of Na$^+$ compared to K$^+$ reduces the mobility, and thus the bulk conductivity, as well as the channel conductance, i.e., 0.7 nS at $+100$ mV and 0.63 nS at $-100$ mV. In the case of MgCl$_2$, despite the high bulk conductivity due to the divalent nature of Mg$^{2+}$ and the presence of two Cl$^-$ ions, the channel conductance was lower, i.e., 0.8 nS at $+100$ mV and 0.75 nS at $-100$ mV. From the MD simulations it can be clearly seen that Mg$^{2+}$ ions bind tightly to the negatively charged residues lining the interior of the channel (see Fig. S2), whereas the ion flow mainly consists of Cl$^-$ ions. This is a nice illustration of the binding affinity of magnesium to the channel interior. The observed asymmetry in conductance might be the result of asymmetric distribution of charged residues across the channel, as shown in Fig. 1 B. We emphasize that the asymmetry observed in bilayer experiments was also reproduced in simulations. However, we noted a relative overestimation of the simulated conductance values for the KCl and NaCl solutions and an underestimation in the case of the MgCl$_2$ solution. This artifact is probably due to the usage of higher voltages and also force-field deficits, although we have already used
improved parameters (38,54). Based on the conductance value, we were able to probe the orientation of the channel in the bilayer, which made it possible to distinguish between the extracellular and periplasmic sides.

Concerning the selectivity of the channel, we determined the zero current membrane potentials, $V_m$, experimentally (Fig. 2 C) and $I_{cation}/I_{anion}$ theoretically (Fig. 2 D). From the positive $V_m$ values in KCl and NaCl solutions, the negative $V_m$ values in MgCl$_2$ solution, and the $I_{cation}/I_{anion}$ values, >1 in the KCl and NaCl solutions and <1 in the MgCl$_2$ solution, one can conclude that the ΔCymA channel is cation selective in KCl and NaCl solutions and anion selective in MgCl$_2$ due to charge inversion. This finding is in accordance with the previously shown inversion of selectivity in the case of divalent ions for other pores (55–57).

Electroosmosis contribution to $\alpha$-CD permeation

Next, we studied the permeation of $\alpha$-CD through ΔCymA. To this end, we performed $\alpha$-CD interaction studies from both sides of the channel using electrophysiology. Due to the high affinity of the CymA channel from the extracellular side, all the experiments were done by adding $\alpha$-CD on the extracellular side. The frequency of the incoming $\alpha$-CD molecules causes ion current blockages and allows us to quantify the association rate, $k_{on}$. The statistical analysis of the length of blockages allows us to evaluate the average residence time, $\tau$ (27,33) (see Materials and Methods). In 1 M KCl at $+100$ mV, complete blockages of the ion current were observed, whereas at $-100$ mV, short-lived transient blockages were seen (as shown in Fig. 3 A). The $k_{on}$ values for $\alpha$-CD differ by a factor of ~2 at the voltages applied, that is, $74 \times 10^6$ M$^{-1}$ s$^{-1}$ at $+100$ mV and $38 \times 10^6$ M$^{-1}$ s$^{-1}$ at $-100$ mV. In contrast, an almost sixfold change in the residence time, $\tau$, for $\alpha$-CD was observed, depending on the polarity of the applied voltage, i.e., 500 $\mu$s at $+100$ mV and 80 $\mu$s at $-100$ mV. The dependence of the EOF on the external electric field should be roughly linear but might be masked by other effects. To elucidate the contribution to the overall flow, the corresponding voltage dependence was investigated. Therefore, we studied the resulting ion-current fluctuations caused by CD molecules for voltages ranging from $-150$ mV to $+150$ mV. Moreover, two additional aqueous environments, 1 M NaCl and MgCl$_2$, were employed in which we were able to modulate the rates severalfold. In KCl and NaCl solutions, similar interactions were seen, whereas in MgCl$_2$, completely different interactions were observed, which can be explained by the anion-selective nature of the pore. Fig. 3 A depicts ion-current traces of CymA with 10 $\mu$M $\alpha$-CD (added to the extracellular side) at $+100$ mV (upper trace) and $-100$ mV (lower trace) in the presence of KCl, NaCl, and MgCl$_2$. Fig. 3 B shows the association rate, $k_{on}$, over a range of applied voltages from $-150$ mV to $+150$ mV in the studied ionic environments. In the case of the salts containing monovalent cations, i.e., KCl and NaCl, we observed a linear increase in the $k_{on}$ rate with increasing positive voltage directed from the extracellular to the periplasmic side.

To gain atomistic insight into the water flow and to complement our experimental results, applied-field MD simulations...
were carried out. The net flux in counts per second was determined from the resulting trajectories (Fig. 3 D). In KCl and NaCl solutions at positive voltages, the ionic current based on the K\(^+\) and Na\(^+\) ions was larger than the one based on the Cl\(^-\) ions. Due to the net cation current and stronger (due to tightly bound water molecules) hydration shell of the cations (see Fig. S3), a voltage-dependent net flow of water in the direction of the K\(^+\) and Na\(^+\) ions was observed. Based on this finding, we strongly suggest that with increasing positive voltages (from the extracellular to the periplasmic side),

**FIGURE 3** (A) Typical ion-current recordings at positive voltage +100 mV *(top row)* and negative voltage –100 mV *(bottom row)* in the presence of 10 μM α-CD on the extracellular *(trans)* side for three buffers, i.e., 1 M KCl *(black)*, 1 M NaCl *(blue)*, and 1 M MgCl\(_2\) *(red)*. *(B and C)* Association rate, \(k_{on}\) *(B)*, and residence time, \(\tau\) *(C)*, of the α-CD molecule as a function of applied voltage are shown corresponding to the current traces in *(A)*. *(D)* The net flux of the cations K\(^+\), Na\(^+\), and Mg\(^{2+}\) and the anion Cl\(^-\) shown as a function of applied voltage. In addition, the net water flux is depicted. *(E)* The schematic representations illustrate the direction of the cations, anions, and water molecules at positive voltages through the ΔCymA pore (for negative voltages, all arrows need to change direction). To see this figure in color, go online.
there is an increase in the $k_{on}$ rate, which implies that more $\alpha$-CD molecules were pushed toward the interior of the channel, which also corresponds to the linear increase in EOF (in the same direction). With increasing negative voltage, a decrease in the $k_{on}$ rate was observed, caused by the fact that the EOF, which follows the direction of $K^+$ and $Na^+$ ions, is in the direction opposite to that of the incoming $\alpha$-CD molecules. In the NaCl solution, as the net number of sodium ions penetrating is lower than that of potassium ions, the resulting EOF should be smaller. However, the EOF estimated for the NaCl salt from MD simulations is quite similar, or at 0.5 V even larger, which might be due to an inaccuracy in the force field for the Na$^+$ ions, although an enhanced version of the force field already has been employed (54). Nevertheless, if one assumes that a smaller ionic current in the case of NaCl results in a weaker EOF, then the number of $\alpha$-CD molecules entering the channel in the case of NaCl would be lower than in the case of KCl, as indicated by $k_{on}$.

In the presence of the divalent-cation-containing salt, MgCl$_2$, $\Delta$CymA is anion selective. As can be seen for MgCl$_2$, the $k_{on}$ rates are much lower than for the KCl or NaCl solutions. Mg$^{2+}$ binds tightly to the negatively charged residues in the interior of the channel, leading to slower diffusion even at higher applied voltages. This strong binding to the channel walls seen in the simulations also rules out larger effects in inaccuracies in solvation shell determinations, as reported earlier. In this scenario, the major ionic current is induced by the Cl$^-$ ions. For positive applied voltages (from the extracellular to the periplasmic side), a decrease in the $k_{on}$ rate with increasing voltage was seen, in which the EOF is directed from the periplasmic side to the extracellular side. Following the movement of Cl$^-$ ions, and opposite to the movement of the Mg$^{2+}$ ions, the EOF had directionality opposite to that for monovalent cations. At negative voltages, a slight increase in $k_{on}$ (up to $-75$ mV) was observed, although the theoretically calculated EOF showed a linear increase in the opposite direction. Although we expect more $\alpha$-CD molecules to approach the channel, drastic changes in the electrostatic potential of the channel in the presence of Mg$^{2+}$ could hinder the effect of EOF at negative voltages. Summarizing all three electrolytes, we demonstrated to a great extent a clear correlation between the $k_{on}$ rate and the net water flux.

The residence time, $\tau$, of CD bound to the channel is shown in Fig. 3 C for different applied voltages. CD spends more time in the channel at positive applied voltages compared to negative ones, i.e., the residence times were higher at positive voltages compared to negative ones in all examined cases. However, the interesting point is that at both voltage polarities, the residence time decreases with increasing magnitude of the voltage. In other words, the residence time has a maximum at or close to vanishing applied fields, which implies that the binding affinity is quite high at the respective binding site in the absence of any external voltage and subsequently gets destabilized by applied fields.

Considering both the $k_{on}$ rate and the residence time, $\tau$, we summarize the effect of EOF on the mode of transport of $\alpha$-CD molecules across $\Delta$CymA. In the cases of KCl and NaCl, an increase in the $k_{on}$ rate and a decrease in the residence time with increasing positive voltage suggests an enhancement in the translocation of $\alpha$-CD with additional electroosmotic drag, whereas a decrease in both $k_{on}$ and residence time at negative voltages clearly suggests the low probability, or possibly rarity, of translocation events, especially at higher negative voltages. For MgCl$_2$, one expects the opposite scenario due to the inversion in the direction of EOF compared to KCl and NaCl. In agreement with our hypothesis, we see a decrease in the $k_{on}$ rate and residence time at positive voltages, leading to reduced translocation events. However, at negative voltages, we observed a steady decrease in the residence time, but only a partial increase, followed by a decay, in the $k_{on}$ rate. A schematic illustration of these EOF-mediated effects on $\alpha$-CD permeation is shown in Fig. 4.

**Atomistic insight into the influence of EOF on $\alpha$-CD binding affinity**

To get an overview of the impact of electrokinetically driven water on the binding affinity of $\alpha$-CD at the entry site of $\Delta$CymA, we have employed unbiased and applied-field MD simulations at $-1$ V and $+1$ V in the absence and presence of 1 M salt solutions. In the absence of ionic salts, as shown in Fig. 5, the $\alpha$-CD molecule shows a high binding stability at the entry site irrespective of the polarity of the external voltage applied. Additionally, the relative mean-square deviation calculated for the $\alpha$-CD bound protein (Fig. S4 A) illustrates no significant deviation of the molecule from the binding site. These simulations clearly indicate the absence of any EOF and the nonappearance of voltage-driven effects on the neutral molecule, $\alpha$-CD, in the absence of ions. Unbiased simulations, i.e., no applied field, carried out in the presence of 1 M KCl or 1 M MgCl$_2$ lead to similar stabilities of the $\alpha$-CD at the entry site of $\Delta$CymA, as shown in Fig. 6 A. Using 1 M KCl or 1 M MgCl$_2$ and applying an electric field, the $\alpha$-CD molecule was easily dislodged from the entry site for both salts, irrespective of the field polarity. From the spatial distribution in Fig. 6 A, one can see that in the presence of 1 M KCl, the molecule tends to localize close to the entry site at positive voltage compared to negative applied voltage. It can be conjectured that at positive voltage, and due to the directive flow of water from the extracellular to the periplasmic side, the $\alpha$-CD tends to localize more toward the mouth of the channel. For the opposite voltage direction, the molecule is pushed away from the binding site. A contrary behavior of the $\alpha$-CD molecule was observed for the
MgCl₂ salt due to the reverse EOF. Moreover, the relative mean-square deviations calculated for all simulations (Fig. S4, B and C) lead to a similar conclusion, i.e., especially in the case of KCl salt, the EOF is larger than for the MgCl₂ salt. Based on these MD simulations, we can conclude that there is a large impact of the water flux in destabilizing the α-CD from its binding site irrespective of the polarity of applied voltage and type of salt used. Obviously, a voltage increase causes an enhancement in the momenta of the ions and the associated water molecules near the channel surface. This enhancement will amplify the force exerted on the α-CD molecule. Therefore, the directed motion of these particles in the presence of an external electric field may decrease the residence time of substrate molecules in the channel even when the EOF is not very effective due to a blocked ion current.

To gain further insight into the EOF on transportation, we performed SMD simulations at 0 V, +1 V, and −1 V. In all simulations, the α-CD molecule was pulled away from the entry site into the bulk toward the extracellular side. We assumed that dissociation of the molecule by pulling into the bulk would give a clearer picture of the role of water compared to pulling through the channel, where the varied electrostatics of the pore, due to the presence of various ions, might affect the externally applied forces drastically. At each voltage, the average force profiles were calculated as a function of distance averaged over five individual simulations. As can be seen in Fig. 6 B, in the absence of an electric field, the force required to displace the molecule was similar for both salts (~180 pN) due to the absence of any EOF. As shown in earlier simulations (Fig. 6 A), we further confirm that the application of an external electric field destabilizes the α-CD from the entry site, which further leads to an approximately two- to threefold decrease in the pulling force, irrespective of the polarity of the electric field and the type of salt used, compared to the case of no applied voltage. Moreover, in the case of the KCl solution, the pulling force required at positive voltage was higher than that at negative voltage, as the molecule has to be dragged against the EOF at positive voltage. Strikingly, the situation is almost reversed in the case of MgCl₂ salt. In this case, the force necessary to pull the α-CD molecule at the negative voltage was higher due to the fact that the EOF has an opposite sign. In these simulations, we clearly find a significant influence of the EOF on the forces needed to pull the substrate away from the entry site.
the binding site. Within a nanosecond simulation timescale, however, we cannot provide a quantitative explanation of the changes in the $k_{on}$ rate and residence time. Nevertheless, these simulations clearly support our above discussion on the strong influence of the EOF on the rate kinetics of neutral molecules in single-channel experiments with respect to different voltages and ionic conditions.

**Electroosmosis-mediated discrimination of translocation versus binding**

The results so far discussed were obtained for $\alpha$-CD as a substrate. Moreover, as a control experiment, we characterized other substrates, such as $\beta$-CD and $\gamma$-CD molecules, with the $\Delta$CymA channel, for which, due to the larger size, we expect no translocation. In these cases, we used 1 M NaCl to facilitate a slow movement of the ions compared to 1 M KCl and hence increase the residence time of the molecules (as shown in Fig. 3 C). The electrophysiological traces in Fig. S5 show an interaction of the three different CDs with the CymA channel. In contrast to $\alpha$-CD, transient short-lived blockages were observed with $\beta$-CD and $\gamma$-CD. The $k_{on}$ rate and residence time are shown in Fig. 7, A and B, respectively, as a function of voltage. With increasing positive voltage, an increase in the $k_{on}$ rate was observed. As can be seen, $k_{on}$ rates were higher for $\alpha$-CD compared to $\beta$- and $\gamma$-CDs, which is assumed to be due to the larger size of the latter molecules leading to slow diffusion. Still, an increase in $k_{on}$ clearly suggests that the electroosmotic drag tends to force the molecules toward the interior of the channel. However, the residence times for $\beta$- and $\gamma$-CDs were lower compared to that for $\alpha$-CD. One would expect a longer residence time for these molecules due to their larger size, corresponding to slower diffusion, compared to $\alpha$-CD, but the estimated low residence times with no voltage-dependent decay give a hint that these molecules undergo no major translocation, unlike $\alpha$-CD at positive voltages. Interestingly, at negative voltages, the $k_{on}$ rates were almost diminished to zero for $\beta$- and $\gamma$-CDs. In addition, the residence time was almost negligible for both molecules compared to $\alpha$-CD. Both of these parameters clearly indicate no significant interaction of these molecules with the $\Delta$CymA channel, which is indeed suppressed by the opposing EOF at negative voltages. With these findings, we conclude that understanding the contribution of EOF in analyzing the single-channel experiment results will help to discriminate major translocation events from binding. A schematic illustration of how this works is shown in Fig. 7 C.

In addition to these measurements, we performed experiments where all three CDs (20 $\mu$M each) were added to the periplasmic side of the channel and the on rate, $k_{on}$, and residence time in 1 M NaCl solution were determined (Fig. S6). Even though a higher concentration of substrate was added (20 $\mu$M on the periplasmic side as compared to 10 $\mu$M on the extracellular side), the on rates were much lower compared to the rates on the extracellular side and an increase in $k_{on}$ was observed with increasing negative voltage. However, the $\tau$ values did not alter significantly with voltage, indicating that the binding affinity from the periplasmic side is considerably lower compared to that on the extracellular side due to the channel architecture.
Nevertheless, these results clearly support the EOF-dependent change in kinetics and shed light on its significance when substrates have strong affinity in a selective channel. α-CD concentration dependence of $k_{on}$ and $t$ in all three salts was studied at both polarities of 100 mV applied voltage with substrate added on the extracellular side (see Fig. S8).

All together, the above results show a strong influence of the EOF on substrate translocation. A water flow can, however, also be caused by a concentration gradient of solutes on both sides of the pore. What is the magnitude of this water flow in comparison to the EOF? To determine this, we performed MD simulations with a hydrostatic pressure difference (49). The derived hydraulic permeability coefficient, $P_h$, from the pressure-driven simulation (Fig. S7) was $44 \times 10^{-17}$ cm$^5$/N$^{-1}$s, leading to an osmotic permeability coefficient of $P_f = 6.1 \times 10^{-12}$ cm$^3$/s for the ΔCymA pore, which is very similar to the value of $5.6 \times 10^{-12}$ cm$^3$/s for the α-hemolysin channel. At +100 mV and with a 10 µM difference α-CD concentration, the computed osmotic flow is $3.6 \times 10^4$ water molecules/s compared to the EOF of $2.3 \times 10^9$ water molecules/s (estimated from Fig. 3 D, left). Thus, under these conditions, the EOF is ~63,000-fold stronger than the osmotically induced water flow. Nevertheless, one should keep in mind that under certain conditions, the osmotic water flow and the EOF can be similar in size.

CONCLUSIONS

To conclude, we have detailed the influence of ion flow in the CymA nanochannel on the transport of water, i.e., the EOF, and its impact on CD permeation. To this end, we have determined the ionic currents in different salt solutions both experimentally and computationally. Passive transport of solvent molecules through a protein channel requires a chemical potential gradient that is in nature the difference in concentration. In electrophysiology, we apply transmembrane electric fields that drive ions across the membrane. An asymmetry in the total ion current induces an EOF and contributes in a nonnegligible manner to the translocation of CD molecules. Due to the sensitivity of electrophysiology in quantifying ion currents, the asymmetry with respect to the applied field can be used to probe for the permeation of uncharged molecules. The presence of solvent molecules inside the channel may block the ion current strongly enough to be resolved by ion-current fluctuations. However, blocking by a substrate without translocation or even solvent-density fluctuations provides signal patterns similar to those provided by transport. Here, we have shown for the case of CymA that the EOF makes an additional contribution to the flow of substrate molecules. As this flow is linear in the external field, we can modulate the hydrodynamic flow and thereby modify the residence time. In our investigation, we compared α-CD as a permeating molecule with β-CD and γ-CD as representative of nonpermeating molecules. Inspection of the residence times of α-CD at positive EOFs reveals shorter residence times driving the molecules faster through the channel with increasing strength of the EOF. The opposite EOF revealed short-lived transient events pulling the CD molecules out of their binding site at the entry site. In contrast, experiments with neutral β- and γ-CD molecules reveal only short residence times. Due to the osmotic pressure, the β-CD and γ-CD molecules were forced to enter...
the channel but due to size limitations and other steric effects, these substrates are unable to bind at the extracellular site. Therefore, these molecules cannot undergo translocation. Likely, they tend to bind but immediately come off the binding site on the same side to which they were added.

In summary, we quantified the nature of the interaction of all three closely related CDs with the CymA channel. Apparently, the EOF has a considerable influence on the transport of these specific neutral molecules through the CymA channel. Having shown this phenomenon for a specific example, it is apparent that this finding will be important for the general case of molecule transport through nanopores. The size of the effect, however, might strongly vary, and its dependence on parameters like size of the channel, charge distribution inside the pore, pH value, ion type, etc., need to be investigated in further studies. To this end, an important first step would be the investigation of the water flow depending on the different parameters. The water flow in turn influences the substrate transport. We emphasize once more that a quantitative understanding of the EOF, especially for neutral molecules, is of importance in distinguishing substrate binding from substrate translocation, a problem that occurs in the interpretation of electrophysiological experiments (33,34,58). This approach obtained for a channel of simple geometry will be applied to the interpretation of antibiotic permeation through pores of bacterial membranes in the future.

SUPPORTING MATERIAL
Eight figures are available at http://www.biophysj.org/biophysj/supplemental/S0006-3495(15)04811-0.

AUTHOR CONTRIBUTIONS
M.W., B.v.d.B., and U.K. designed the research; S.P.B. and M.W. performed and analyzed the electrophysiology experiments; J.D.P. and U.K. performed and analyzed MD simulations; and S.P.B., J.D.P., B.v.d.B., M.W., and U.K. wrote the article.

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