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Increased salt concentration promotes competitive block of OmpF channel by protons

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ABSTRACT

Porins are channel-forming proteins that are located in the outer membranes (OM) of Gram-negative bacteria and allow the influx of hydrophilic nutrients and the extrusion of waste products. The fine regulation of the ion transport through these wide channels could play an important role in the survival of the bacteria in acidic media. We investigate here the mechanism responsible for the pH sensitivity of the trimeric porin OmpF, of *Escherichia coli*. Planar lipid bilayer electrophysiology and site-directed mutagenesis were used to study the effect of pH on the ion conductive properties of the OmpF channel in its fully open, "nongated" conformation. At low pH we observe a large drop in the OmpF open channel conductance that is accompanied by a substantial increase of the current noise. These channel features are strongly dependent on the salt concentration and we propose that they are originated by competitive binding of cations and protons occurring in the narrow central constriction of the channel. This subtle mechanism reveals to be capital for the channel function because it not only drives the channel sensitivity to pH but is also indispensable for the particularly efficient permeation mechanism of the channel at physiological conditions (~neutral pH).

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1. Introduction

Ion channel function is crucial for neural transmission and key physiological processes in the cardiac, pulmonary and muscle systems. These specialized physiological functions require sophisticated control of the transport mechanisms through channel pores so that the exchange of ions, metabolites and other larger molecules across the cell membrane could be accomplished at the precise rate [1,2]. Much attention has been paid to explore the role of pH, which is a well-known modulator of channel activity in a variety of natural habitats [3–5]. Indeed, protons regulate the single channel conductance of many channels like K^+, Na^+, CNG, BK and Ca^{2+} channels, among others [6–9]. The sensitivity to pH may be particularly critical for porins (such as OmpF, OmpC or PhoE) located in the OM of Gram-negative bacteria, such as *E. coli*, that can survive in inhospitable environments like the stomach with pH as low as 1.5–2.5 [4,5]. The total or partial inactivation of wide porins located in the OM may take part in protecting bacteria against acidic media [10].

We study here the effect of salt on the pH titration of the OmpF porin [4,11,12], focusing on single channel conductance and current noise. OmpF forms trimeric channels [13], so that each monomer is identical and functionally independent [14,15] (Fig. 1). It has been reported that in OmpF, an increase of the applied voltage (>150 mV) leads to a sequential step-wise closure of the channel monomer and this effect can be enhanced by low pH. We do not address here such high voltage-induced channel gating [16]. On the contrary, we restrict ourselves to relatively low voltages (≤100 mV) to investigate the mechanisms by which pH modulates the function of the fully open channel. Protons can regulate channel conductance by simply blocking the channel but they can also exert more fine-grained control by titrating essential residues within the pore [1–3,17,18]. These molecular mechanisms are poorly understood because of the complex interplay between structural factors and physicochemical phenomena, such as networks of titratable residues, inter- and intramolecular interactions and regulatory interfaces [3]. Previous theoretical and experimental studies have suggested the existence of a binding site for cations in the central narrow constriction of the OmpF channel [19–22]. By combining different experimental techniques: site-directed mutagenesis, electrophysiology and noise analysis, we show that a competitive interaction involving both protons and cations is likely to exist. Such interaction not only drives the channel sensitivity to pH but is also indispensable for the particularly efficient permeation mechanism occurring at physiological conditions (~neutral pH).

2. Material and methods

2.1. Ion channel recording

Isolation and lab handling of OmpF channels was analogous to the method described elsewhere [15,23,24]. Wild-type OmpF, kindly provided by Dr. S. Bezrukov (NIH, Bethesda, USA), was isolated and purified from an *E. coli* culture. Mutants D113C and D113C/E117C [25] were a generous gift from Dr. H. Miedema (Wetsus, The Netherlands). Planar membranes were formed by the apposition of monolayers [26] across orifices with diameters of 70–100 μm on a 15-μm-thick Teflon partition using diphytanoyl phosphatidylcholine. The orifices were pre-treated with a 1% solution of hexadecane in pentane. An electric potential was applied

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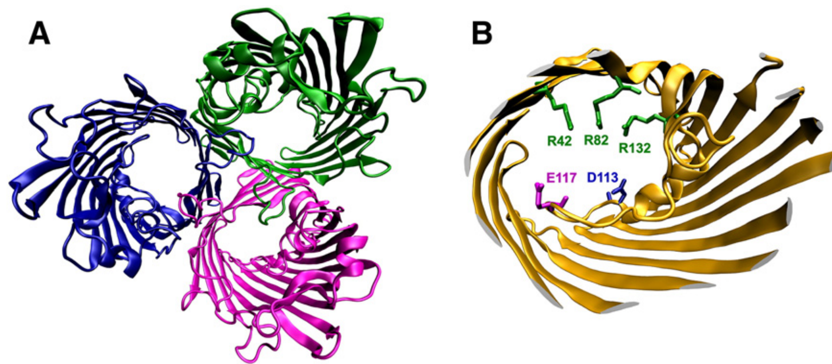

Fig. 1. Ribbon representations of the OmpF channel obtained from the crystal structure (PDB ID: 2OMF). A) The three monomers that assemble to form the OmpF channel behave as independent subunits and display identical permeation properties. Each beta-barrel structure opens an aqueous pore that allows the permeation of charged and neutral solutes. B) The channel constriction (approximately 0.7×1.1 nm) created by loop L3 halfway along the channel axis [13]. Two acidic residues (D113 and E117) and three basic residues play critical roles in the regulation of ionic channel conductance.

using Ag/AgCl electrodes in 2 M KCl, 1.5% agarose bridges assembled within standard 250 ml pipette tips. The potential was defined as positive when it was higher on the side of the protein addition (the *cis* side of the membrane chamber), whereas the *trans* side was set to ground. An Axopatch 200B amplifier (Molecular Devices, Sunnyvale, CA) in the voltage-clamp mode was used to measure the current and applied potential. The chamber and the head stage were isolated from external noise sources with a double metal screen (Amuneal Manufacturing Corp., Philadelphia, PA). The pH was adjusted by adding HCl or KOH and controlled during the experiments with a GLP22 pH meter (Crison). Except where noted, measurements were obtained at $T=(23\pm1.5)^{\circ}$C. The average open channel conductance was obtained from the current measurement at an applied potential of ±100 mV in symmetrical salt solutions. The low-frequency current spectral density, S(0), was obtained from a single-Lorentzian approximation of the ion current power spectra, as described elsewhere [15].

2.2. Fitting the experimental data

The titration curves in Fig. 2 contain information about the underlying physicochemical processes in the proton–protein interaction [27]. According to the Hill formalism, used in ligand–receptor binding [28], the inhibition of the channel conductance by protons can be described by Eq. (1):

$$\frac{g}{g_{\max}}=\frac{K^n}{K^n+[H^+]^n} \tag{1}$$

where $[H^+]$ is the concentration of protons, K is the inhibitory constant of the blocker and n is a Hill coefficient. This equation can be alternatively expressed as a function of solution pH following the treatment of Rostovtseva et al. for VDAC channel [29]:

$$g=g_{\min}+\frac{g_{\max}-g_{\min}}{\left(1+10^{n(pK-pH)}\right)} \tag{2}$$

where the effective pK is the pH that provokes a response halfway between the baseline (g_{min}) and the maximum (g_{max}) conductance. Again, the dimensionless Hill coefficient n describes the steepness of the curve. Values of $n<1$ correspond to shallower curves, and values of $n>1$ correspond to steeper curves. A similar equation (known as Bezrukov–Kasianowicz equation) was obtained for the low-frequency current spectral density using Machlup's original derivation for random telegraph signals [29–31]

$$S(0)=\frac{4(\Delta i_{\max})^2 10^{n(pK-pH)}}{k_{off}\left(1+10^{n(pK-pH)}\right)^3}. \tag{3}$$

The ionization-induced change in the average current through a single channel Δi is a strong function of pH (see current traces in Fig. 2C). Following the treatment described in [29], we obtained values of k_{off} for the inverse of the characteristic time of subconductance states and Δi_{max} for the average change in the current between states of maximum conductance and the substate.

3. Results

3.1. pH modulation of OmpF channel function

The measured single channel conductance was strongly dependent on the acidity of the solution, as shown in Fig. 2A [11,15,32]. Eqs. (1) and (2) are fully equivalent and fitting the conductance measurements to any of the two equations yields the same values of the free parameters n and pK. However, the latter is more convenient for the fitting, so that we chose Eq. (2), in which the relation between conductance and $[H^+]$ was expressed as a function of solution pH. The solid lines through the data points in Fig. 2A and its inset are the best fit plots of conductance according to Eq. (2).

Compared with its value at neutral pH, the channel conductance at low pH was reduced by a factor of almost 7 in 2 M KCl, (Fig. 2A) and a factor of 2 in 0.5 M KCl (inset of Fig. 2A). It is important to stress again that all measurements reported here correspond to the channel conductance in its fully open, "nongated" conformation. As shown in Fig. 2C, the single-channel current traces analyzed here do not display the characteristic three step voltage-induced closures of OmpF [14], because they were recorded at low enough voltages to avoid such gating.

Fig. 2B shows the OmpF current noise intensity at low frequencies, or "zero-frequency spectral density", S(0), as a function of pH. The solid line is the best fit of the data to the model Eq. (3). It shows a pronounced and clearly defined peak of current noise at pH 2.5. This high fluctuation peak corresponds to the pH region where the most significant decrease in single-channel conductance occurred. Interestingly, in less concentrated solutions (0.5 M KCl) a smaller reduction of conductance was found (inset of Fig. 2A), and only a small (two orders of magnitude lower than in 2 M KCl) peak of current noise appeared near pH 3 (inset of Fig. 2B). This finding suggests that the effect of salt is the opposite of the well-known salt-screening effect [23]: here, increasing salt concentrations enhanced the interactions responsible for the conductance drop.

Two facts become apparent from the fittings of conductance and zero-frequency spectral density measurements reported in Fig. 2. First, the best fit for the Hill coefficient n was always less than 1 and decreased with increasing salt concentration. Fixing the Hill coefficient to 1.0 gave considerably worse fits of the data (not shown here). Second, the best fit for the effective pK of the titration curve decreased

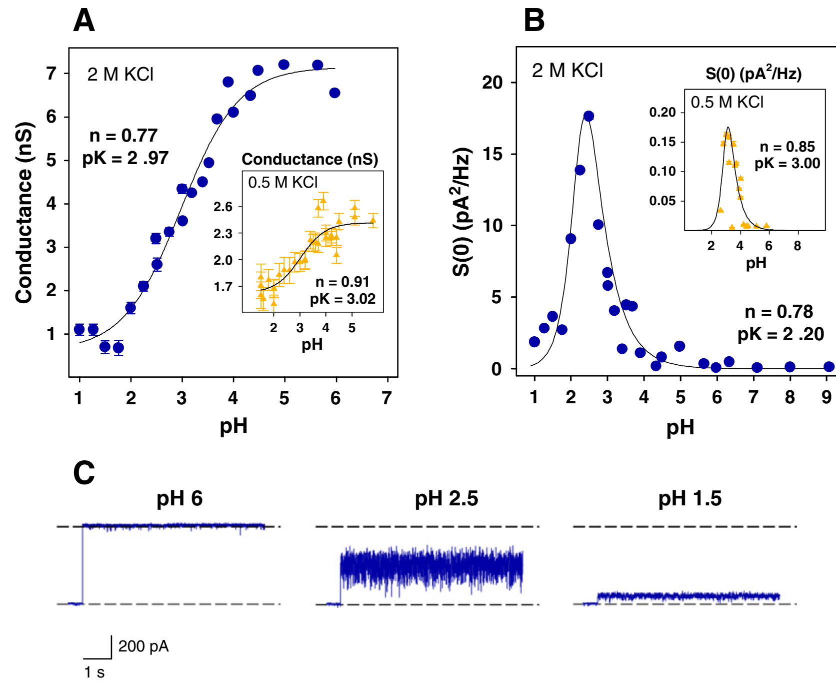

Fig. 2. OmpF conductance is modulated by pH. A) The measured single-channel current was strongly dependent on the concentration of protons in the solution, as seen in the large drop in conductance at low pH. The main plot shows the channel average conductance in 2 M KCl, and the inset displays the weaker conductance decrease in 0.5 M KCl. B) Low-frequency spectral densities of OmpF current fluctuations in 2 M KCl solutions strongly depend on solution pH and exhibit a sharp peak at about pH 2.5. This effect decreases considerably in 0.5 M KCl solutions. C) Typical single-channel current traces measured in 2 M KCl at neutral pH, at pH 2.5 and pH 2.5. Conductance decreases monotonically but current fluctuations peak around pH 2.5.

with increasing salt concentrations. In addition, all effective pKs in these concentrated solutions were anomalously low in comparison with the apparent pK ca. 4 reported in 0.1 M KCl solutions [15], which is comparable to nominal pKs of glutamic and aspartic acid, the putative residues responsible for OmpF channel titration, as will be discussed later.

3.2. Proton block of OmpF channel is salt dependent

Classic theories of membrane permeation (electrodiffusion models and kinetic rate theories) rely on the concept of ion independence: fluxes of one ion are assumed not to change as the concentrations of other ions are varied [33,34]. Deviations from independence may lead to current saturation and blocking of channels with changing ion concentrations [1]. Having in mind previous experiments indicating that low pH does not induce conformational changes in OmpF [15], we can wonder whether the origin of the huge reduction (>85%) of channel conductance at low pH could involve some kind of interaction that hinders the independence of ion fluxes. Fig. 3 shows the measured channel conductance as a function of both pH and salt concentration.

In the range pH 4–8, the observed channel conductance scaled with salt concentration as expected under current independence conditions. However, the situation changed gradually in solutions of increasing acidity. Below pH 3.5 the change in conductance with salt concentration becomes increasingly sublinear. In the range pH 4–8, a four-fold increase in salt concentration (from 0.5 M to 2 M) increases channel conductance by 300%. In contrast, in the range pH 1.5–2, the same increase in concentration only yields a 50% increase in channel conductance.

3.3. Competitive interaction between H^+ and K^+

Having in mind the huge effect of salt concentration on the channel block by protons, we can investigate whether the interaction might be consistent with a competitive or a noncompetitive blocking model. We use here an approach originally derived to study enzyme kinetics [35] that was later adapted to the block of ion channels [9,36]. A simple test can be done by using double reciprocal plots of channel conductance and salt concentration, as shown in Fig. 4A. For a competitive block, the results with and without blocker intersect on the y-axis, meaning that at infinite concentration of K^+ the blocker (H^+) has no effect. In contrast, for a noncompetitive block the results intersect on the x-axis, indicating that regardless of the concentration of K^+, the block of the channel by H^+ cannot be relieved. To examine the nature of the proton block, Fig. 4B presents double reciprocal plots of single channel conductance in three different pH conditions. In the case of pH 6 and pH 2.5 the intersection of the lines in the y-axis is consistent with that of a

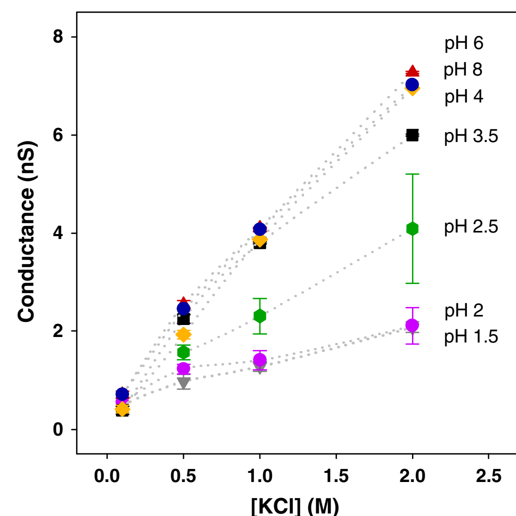

Fig. 3. The increase in OmpF single channel conductance with KCl concentration changes considerably in highly acidic solutions. Below pH 3, the channel conductance is much less responsive to an increase in salt concentration of the bathing solutions. This reduced efficiency in the channel ability for transport of small inorganic ions suggests a loss of independence between the currents carried by the salt ions and protons.

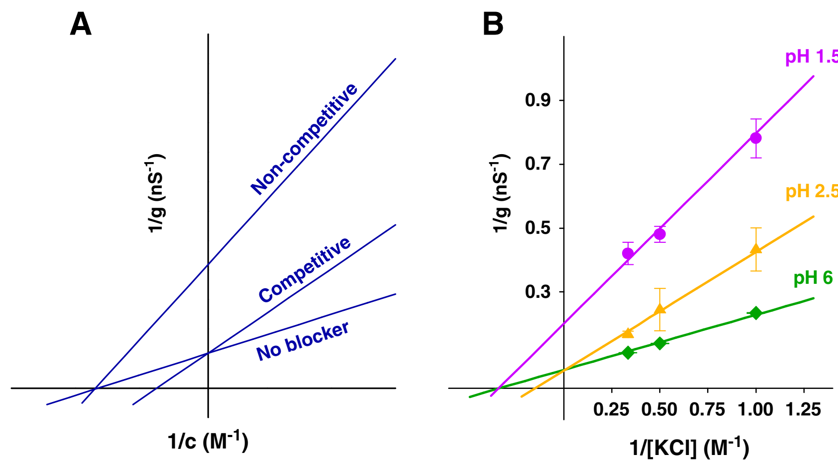

Fig. 4. Proton block of OmpF channel can be described as a competitive interaction between H^+ and K^+. A) Double reciprocal plot of theoretical conductance versus $[K^+]$ to illustrate competitive and noncompetitive block [9]. B) Double reciprocal plot of measured single channel conductance versus $[K^+]$ at pH 6.0 (no blocker), pH 2.5 and 1.5 (high concentration of the blocker). The intersections with the axis of the regression lines at pH 2.5 and 1.5 are consistent with a competitive and noncompetitive block, respectively.

competitive block. Interestingly, when the acidity is increased to pH 1.5, the nature of the block changes to noncompetitive. The amount of protons is so high that the binding sites are protonated no matter how much cations fill the channel solution. This is consistent with the current traces shown in Fig. 2C. At pH = 1.5, the noise is similar to pH = 6 so that the intense flickering characteristic of the competitive binding (pH 2.5) dies out.

3.4. Residues involved in the block of OmpF channel

In a previous study reporting pH-dependent fluctuations in current through the OmpF channel [15], conductance histograms of the transient flickering to lower subconductance states suggested that at least two different residues were involved in the abnormal current noise. Nestorovich and colleagues proposed that the observed low-pH-induced flickering could be explained by the protonation of the aspartic acid D113 and the glutamic acid E117, located at the pore eyelet (see Fig. 1B). To test this explanation, we performed conductance experiments using mutant forms of the protein that differed at these crucial residues (Fig. 5). For the sake of comparison, the results are shown together with the conductance measured in wild-type (WT) OmpF already shown in Fig. 2A. The substitution of the neutral cysteines (CC-mutants) for the two acidic residues D113 and E117 partially eliminated the large conductance decrease found for WT OmpF in low pH solutions (a conductance drop by a factor of seven with respect to the neutral pH value). Indeed, the CC-mutant conductance at low pH is only around half the value at neutral pH. This finding supports the hypothesis that the D113 and E117 residues are strongly involved in the low-pH-induced OmpF current reduction. It is important to note that neither the conductance measurements with the CC mutant shown in Fig. 5, nor the corresponding current noise amplitude displayed a dependence on pH strong enough to allow a reliable analysis based on the Hill formalism. Interestingly, the aspartic D113 is likely to contribute more to the pH-induced OmpF current drop, as follows from the observation that the conductance of mutant D113C was similar to that of the CC-mutant. In contrast, the E117C mutant conductance (data not shown) was intermediate between those of the WT OmpF and the D113C mutant.

4. Discussion

The experiments reported so far indicate that protons exert a crucial regulation in the transport properties of the OmpF channel controlling the channel conductance and its selectivity [15,23].

The reduction of the channel conductance observed at acidic pH (Fig. 2A) could seem counter-intuitive because the addition of protons would normally be expected to increase the conductance. However, there is no such general trend in porins; exhaustive experiments have shown that as the pH is decreased, the channel conductance in various porins either increases (e.g. OmpU of *Vibrio cholerae*, α-toxin of *Staphylococcus aureus*, VDAC of *Neurospora crassa*), decreases (TolC of *E. coli*, OmpT of *V. cholerae*) or remains constant (OmpC of *E. coli*) [18,37].

A variety of explanations have been proposed to describe the observed conductance drop at acidic pH in OmpF. Initially it was ascribed to the formation of alternative porin conformations with smaller channel sizes [18]; however, more recent polymer-partitioning experiments suggested that the pore diameter is not reduced but remains almost unaltered in acidic conditions [15]. Another possible reason, the screening effect of the lipid charges [9], can be discarded here because all experiments have been carried out in neutral lipids.

The most plausible reason so far is a proton block coupled in some way to the presence of salt, which reduces the channel conductance. Measurements performed at the single channel level shown in Fig. 2 demonstrate that increasing salt concentration promotes shallower titration curves with lower effective pKs. A similar phenomenon has

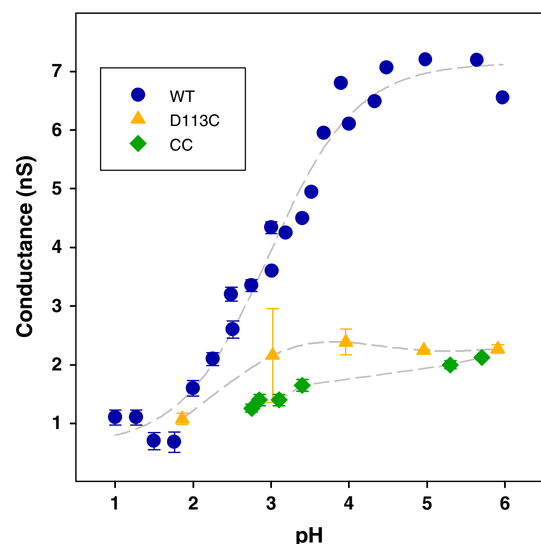

Fig. 5. The pH titration of the OmpF average single-channel conductance differed considerably between the WT and mutants in which one (D113C) or two (D113C/E117C, labeled as CC) of the acidic residues in the channel constriction were replaced by neutral cysteines. The experiments were performed in 2 M KCl solutions. Dashed lines are shown to guide the eye.

been recently reported for divalent cations just in OmpF [38]. Such proton block would provide acid sensitivity over a larger pH range at the price of giving a less decisive response. It is tempting to speculate if this sophisticated control of the channel conductive properties might participate in the survival of the bacteria in acidic media.

In addition to the experiments reported in Fig. 2 (conductance and low frequency noise), the coupling between K^+ and H^+ is supported by the data displayed in Fig. 3, which shows the dependence of channel conductance on salt concentration, for different pH values. The deviation from linearity for pH 3 and below reveals the loss of independence of ion fluxes [1] thus indicating an interaction between the two types of ions, H^+ and K^+.

The block of ion channels can be described by a variety of different mechanisms. Some of them involve the simultaneous presence of protons and salt cations in a non-competitive manner: protons could simply neutralize the channel negative residues avoiding the accumulation of cations near the pore walls and yielding a reduction of conductance [8,9,39]. However, this possibility is not easy to reconcile with the fact that the effective pK of titration curves actually depend on salt concentration (Fig 2A). In addition, a screening mechanism should not increase the open channel noise, in contrast to the data from Fig. 2B and C.

By using double reciprocal plots of the measured single-channel conductance versus $[K^+]$ we show that increasing concentration of the substrate fully relieves the proton block (Fig. 4). This indicates that a plausible mechanism for the blocking would be a competitive interaction involving both cations and protons. K^+ competes with H^+ either directly or indirectly via an allosteric mechanism [9], so that the presence of cations alters the protonation of the acidic groups requiring an abnormally high amount of protons to effectively neutralize the site yielding a lower effective pK (see Fig. 2A). This would also explain why the protonation of some specific residues is extremely noisy and the shape of the spectral density is strongly dependent on salt concentration (see Fig. 2B).

Site-directed mutagenesis allowed for the identification of the residues involved in proton/cation binding. The replacement of the acidic residues D113 and E117 with two neutral cysteines (CC-mutants) changes radically the sensitivity of the channel to acidic conditions, in agreement with previously reported selectivity experiments [22] The fact that the sole substitution of two residues (out of 102 ionizable ones) yields so strong an effect suggests that both D113 and E117 have a hand in the pH sensor of the channel. Besides, two additional features deserve further discussion. First, at neutral pH the channel conductance of the CC mutant was almost one-third of that of WT OmpF. Similar observations were previously reported by Phale et al. in the D113N/E117Q [40]. Second, it is worth noting that the WT, CC-mutant and D113C mutant all displayed nearly identical single channel conductances at pH 2. Both facts seem to indicate that the residues D113 and E117 participate in a particularly efficient permeation mechanism active around neutral pH, so that when they are either mutated or protonated the channel conductance drops dramatically.

The OmpF channel has an hour-glass shape with a narrow constriction located about half of the channel total length. Fig. 1B displays a schematic view of the constriction zone, showing the two acidic residues (D113, E117) that face a cluster of three positive arginines (R42, R82, R132). At neutral pH, all these five residues are ionized and create a high transverse electric field ($E \sim 10^9$ V/m) [41–43] in the channel eyelet. According to MD simulations this electric field gives rise to a peculiar and effective permeation mechanism in which cations and anions follow well-separated permeation trajectories along the pore in a screw-like fashion [44]. Anions cross the eyelet close to the positively charged residues whereas the cation pathway runs near the negative ones. When these key acidic residues are neutralized (via mutation or proton titration) the transverse electric field almost vanishes and the transport mechanism is greatly distorted. The constriction of the channel is then dominated by a cluster of three positive charges that hinder the transport of cations yielding a significant reduction of the overall conductance. This decrease is consistent with the three times difference in conductance between WT-OmpF and the CC mutant.

5. Conclusions

We analyzed the effect of pH on OmpF channel function with a particular emphasis on the huge reduction of the channel conductance found in acidic conditions. We suggest that the molecular mechanism that gives rise to this fine-grained sensitivity to the acidity is a competitive binding between protons and cations. We show that these interactions broaden the channel response to the acidic stress to allow a channel response that is effective over a wide pH range (from pH 4 to pH 1 in some cases). Site-directed mutagenesis indicates that D113 and E117 residues play a decisive role not only in the channel sensitivity to pH but also in the regulation of ion transport at physiological conditions (around neutral pH).

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