Research Paper

Modulation of Vibrio cholerae Porin Function by Acidic pH

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ABSTRACT

The outer membrane of Gram-negative bacteria contains porins, large pore-forming proteins which allow the traffic of hydrophilic compounds between the external medium and the periplasm. The oral mode of infection of Vibrio cholerae, the agent of cholera, implies that the bacteria must adapt to severe changes in the environment, such as acidic pH and the presence of bile. Because of their localization and the regulation of their expression in response to these external factors, the OmpU and OmpT porins of V. cholerae are thought to be involved in the adaptation of the bacteria to the host environment. Using patch clamp and planar lipid bilayer electrophysiology, we assessed the effect of pH on the channel properties of OmpU and OmpT. OmpT does not show any major modification in its activity between pH 4 and pH 7.2. In the case of OmpU, the effect of acidic pH is manifested by promoting single-step closures, whose duration, frequency and current size increase as pH is lowered, thereby producing a pH-dependent decrease in the channel open probability. Surprisingly, the increase in current size of this single-step closure is not coupled with an increase of the total current through the porin, indicating that the trimeric conductance remains unchanged. This observation suggests that coordinated events take place at the level of the trimer, and various explanations for this peculiar effect of acidic pH on porin gating and conductance are provided.

INTRODUCTION

Vibrio cholerae is a Gram-negative pathogenic bacterium and the agent of cholera. It naturally lives in aquatic ecosystems but can infect humans upon oral ingestion of contaminated food or water. Before colonizing the intestinal epithelium, the bacteria have to face severe changes of the external conditions upon entering the host, including acidic pH and the presence of bile salts. Therefore, the bacterium must sense these changes in order to respond and to survive.1 Bile, temperature, osmotic stress as well as pH have been shown to modulate the expression of ToxT and the activity of ToxR.2 These two proteins play a major role in controlling the production of virulence factors, such as cholera toxin; in addition, ToxR controls the expression of the two general diffusion porin genes ompU and ompT.3-7 Once inside the host, the activation of ToxR is believed to turn on ompU expression while repressing ompT expression. Although OmpU and OmpT are not required for virulence,8 the type of porin present in the outer membrane may have an impact on the ability of V. cholerae to colonize the duodenum. For instance, cells have a higher sensitivity towards deoxycholic acid, a major bile component, when solely OmpT is present in the outer membrane than when solely OmpU is expressed.8 This may be due to a facilitated translocation of deoxycholic acid through OmpT relative to OmpU.9

Porins are transmembrane proteins located in the outer membrane of Gram-negative bacteria where they form channels.10 This location confers them with a primary role in interacting with the environment; indeed porins are mostly responsible for the flow of hydrophilic compounds, nutrients or environmental signals that reach the periplasm from the external medium. In V. cholerae, the pore forming properties of OmpU and OmpT have been demonstrated by liposome swelling assays,11 antibiotic flux assays12 and electrophysiological studies.13 The electrophysiological behaviors of OmpU and OmpT are typical of porins, but bear distinct properties. They are both in a highly open state at neutral pH and low transmembrane potentials, but are inactivated by high voltages. OmpU is more resistant to high potentials than OmpT, with an inactivation threshold occurring around 180 mV, while OmpT starts inactivating at 90 mV. In addition, OmpU is more cation selective than OmpT. The sizes of the pores of OmpU and OmpT are

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NOTE

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not known since no structure has been established for any of these porins. The translocation of various compounds through these porins has been assessed, but taken altogether, the results from various studies do not allow any clear-cut conclusion to be drawn so far. For example, OmpU allows the passage of larger sugars than OmpT does,\textsuperscript{11} whereas the deoxyncholic acid molecule is only able to enter and inhibit the OmpT pore but not OmpU.\textsuperscript{9} In vivo, the antibiotic flux measured through OmpT is greater than the one measured through OmpU.\textsuperscript{12} OmpU’s high sequence homology to OmpF, a homo-trimeric \textit{Escherichia coli} porin, permitted the construction of a structural model for OmpU, which, as for OmpF, displays a 16 stranded \( \beta \)-barrel with long loops on the extracellular side. However, a prediction model of OmpT estimates a secondary structure with only 14 \( \beta \)-strands, similar to the monomeric porin OmpG.\textsuperscript{14} Both OmpU and OmpT appear to assume a trimeric quaternary structure, as do the \textit{E. coli} general diffusion porins OmpF and OmpC. The molecular weight of native OmpU was estimated to be 110 kDa, about three times that of heat-dissociated monomers, by size exclusion chromatography.\textsuperscript{11} Native OmpT migrates as an oligomer on SDS-PAGE gels, and shows multiple high molecular weight bands (\( \sim 100 \text{ kDa} \)) typical of porins with tightly bound LPS;\textsuperscript{13,15} it also shows gating to three well-defined levels, corresponding to each monomer, at high voltages (see below) or when blocked by deoxyncholic acid.\textsuperscript{9}

The effect of acidic pH on porins has been previously described in different organisms. In OmpF, low pH affects many electrophysiological properties of the channel. Nestorovich and colleagues showed that it increases the frequency of substate events and decreases the ion selectivity of the porin.\textsuperscript{16} According to the authors, these effects may be due to the titration by pH of residues E117 and D113 of the constriction zone of the pore. Acidic pH also modulates the porin activity by enhancing the frequency of channels closures, driving OmpF to close all three monomers. This is hypothesized to be caused by conformational changes induced by pH in the external loops of the porin.\textsuperscript{17} Experiments performed on another \textit{E. coli} porin OmpC revealed that acidic pH stabilizes the porin in a closed state without affecting the single channel conductance.\textsuperscript{18} OmpG, a monomeric porin of \textit{E. coli} also displayed pH induced modulation in acidic conditions, with an increased frequency of the single step transition between the open and the closed state.\textsuperscript{19} The properties of TolC, a unique trimERIC protein with an outer membrane \( \beta \)-barrel core and a \( \alpha \)-helical periplasmic tunnel domain,\textsuperscript{20} have also been investigated at various pHs, and acidic pH was found to cause a decrease in channel conductance.\textsuperscript{21} Another interesting effect of pH has been observed on the channel formed by the \( \alpha \)-toxin of \textit{Staphylococcus aureus}.\textsuperscript{22} This peptidic exotoxin assembles as homooligomers in biological membranes and form large pores, which remain open most of the time at neutral pH. When the pH is dropped to 4.5, the closing probability of the channel increases, although the mean current measured through the open pore for a given voltage is increased by acidic conditions. In addition, the characteristic noise of the open state of the channel is greater when the pH is lower. Bezrukov and colleagues explain these effects by binding of protons to ionizable residues inside the channel. The electrostatic potential profile would be affected by proton binding, causing a stepwise increase in the current carried by the electrolytes.\textsuperscript{22}

Using electrophysiology, we describe here the effect of acidic pH on the two major porins of \textit{Vibrio cholerae} OmpU and OmpT. As we have documented for other parameters in our previous publications,\textsuperscript{9,13} the two porins again show distinct responses. In addition, the response of OmpU, the most sensitive of the two, is quite different from that of the \textit{E. coli} homolog OmpF.\textsuperscript{16,23-25}

**MATERIALS AND METHODS**

**Chemicals, media, and buffer composition.** The ompU or ompT genes were expressed from a pBAD30 plasmid in the porin-deficient \textit{V. cholerae} strain KKV884.\textsuperscript{8,13} Cells were grown in Luria-Bertani broth (1% tryptone, 1% NaCl, and 0.5% yeast extract) with appropriate antibiotics (0.1 mg/ml ampicillin, 0.1 mg/ml streptomycin) and L-arabinose (0.01% for ompT expression, 0.05% for ompU expression). Tryptone and yeast extract were from Difco Laboratories.\textsuperscript{26} \( N \)-Octyl-oligo-oxyethylene (octyl-POE) was purchased from Axxora. The pentane and hexadecane used in planar lipid bilayer experiments were from Burdick & Jackson and TCI, respectively. The diphytanoyl-sn-glycero-3-phosphocholine was from Avanti Polar Lipids, Inc. Other chemicals were from Sigma. The following buffers were used for the electrophysiological studies: buffer A (150 mM KCl, 10 \( \mu \)M CaCl\(_2\), 0.1 mM K-EDTA, 5 mM HEPES pH 7.2), buffer B (Buffer A + 20 mM MgCl\(_2\)), and a buffer series which are essentially as buffer A but with pH's ranging from 4 to 9.2, as indicated in the figures. For buffers at pH\_5.2, HEPES was substituted by MES. For buffer at pH 9.2, HEPES was substituted by CHES.

**Protein purification.** OmpU and OmpT proteins were purified as previously described.\textsuperscript{13} The outer membrane was isolated by high speed centrifugation (340,000 \( \times \) g) after two French press treatments of the cells at 14,000 psi. The porins were then extracted at 4°C using the detergent octyl-POE. An anion exchange chromatography on a MonoQ RH 10/10 (Amersham Biosciences) followed the extraction. The protein eluted between 130 and 210 mM NaCl (in 1% octyl-POE, 10 mM sodium phosphate, pH 7.6). The OmpT- or OmpU-containing fractions were further purified by size exclusion chromatography on a HiLoad 26/20 Superdex 200 prep grade column (Amersham Biosciences) in 1% octyl-POE, 50 mM NaCl, 10 mM sodium phosphate buffer (pH 7.6). Proteins were identified by Western Blot, and their purity was assessed by silver staining after SDS-PAGE. The trimeric structure of the purified proteins was assessed by comparing the SDS-PAGE migration pattern of boiled and unboiled samples in SDS, as described.\textsuperscript{13} For long-term storage, the samples were kept at 80°C in the elution buffer. The bicinchoninic assay (Pierce) was used to determine protein concentration.

**Reconstitution into liposomes for patch-clamp electrophysiology.** Patch-clamp experiments were performed on unilamellar blisters induced in buffer B from giant liposomes containing the porin, as described.\textsuperscript{13,26} Pure porins were reconstituted into soybean phospholipids (Azolecint, from Sigma) at protein-to-lipid ratio of 1:3,000 to 1:5,000 (w/w). Patches were obtained with \( \sim 10 \) megohm resistance pipettes filled with buffer A and brought into contact with the blister membrane to generate seals of 0.5–1.0 gigaohm. After seal formation, the patch was excised by a brief exposure to air and the bath solution was exchanged for the appropriate buffer. In all patch-clamp experiments, the pipette solution was buffer A (pH 7.2), and the bath solution was buffer A or a buffer at a different pH. All solutions were filtered through a 0.2 \( \mu \)m filter before use.

**Channel reconstitution in planar lipid bilayers.** For insertion experiments, two chambers were assembled, separated by a 0.01 mm thick Teflon film pierced with a 100 \( \mu \)m hole. The hole was pretreated with a 1% solution of hexadecane in pentane. The chambers were filled with \( \sim 2 \) mL of the appropriate buffer, and thus the pH was...
Electrophysiological data recording and analysis. Currents were recorded with an Axopatch 1D amplifier (Axon Instruments) using a CV-4 headstage for patch-clamp experiments and a CV-4B headstage for planar lipid bilayer experiments. The current was filtered at 1 KHz, digitized at 1.25 ms sampling intervals (ITC-18, Instrutech), and stored on a PC computer using the Acquire software (Bruxton). For patch-clamp experiments, the voltages given correspond to pipette voltages. In bilayer experiments, the trans-side was grounded, and the voltages given correspond to those of the cis-side. Data analysis was performed with a program developed in the lab and written by Arnaud Baslé using Microsoft Visual Studio C.net, and with the Clampfit 9.0.1.07 program from Axon Instruments. The open probability Po was calculated as the ratio of the observed integrated current obtained over a 1-minute long recording to the total current expected for the same duration if the current value remained at the fully open level. Average open or closed times were obtained with Clampfit. The fit of the pH dependence of Po and conductance was done with SigmaPlot (Marquardt-Levenberg algorithm) using the following equation:  

\[ Y = Y_{\min} + (Y_{\max} - Y_{\min}) \times \frac{[10^{(pH-pK)}]}{[1 + 10^{(pH-pK)}]} \]  

where Y is conductance or Po, and Y_{\min} and Y_{\max} are 0 and 1, respectively for the Po fits, and the conductance at the minimum and maximum plateaus, respectively, for the conductance fits.

**RESULTS**

Behavior of OmpU and OmpT at neutral pH. Before documenting the effect of pH on porin activity, we will first describe some of the electrophysiological properties of the *V. cholerae* porins OmpU and OmpT at neutral pH using the patch-clamp technique (reviewed in ref. 13). Figure 1 shows representative traces of the spontaneous and voltage-induced activity of OmpU and OmpT at pH 7.2. At low voltages, OmpU exhibits a very quiet kinetic behavior. The trace displayed in Figure 1A shows a 30 sec recording from a patch containing a single trimer, during which the current remains mostly at the fully open level (marked “O”) except for a few very transient closing events (downward spikes). These spikes indeed represent channel closing, as exemplified in Figure 1B, where the 1 sec segment marked by the asterisk above the trace of Figure 1A is displayed on an expanded time scale. The average dwell time of these events at pH 7.2 is 10.7 ± 1.4 msec. The average size of these closing events is 22 pA (at +70mV), which corresponds to a conductance of 314 pS. This conductance is equal to about one third of the full conductance of a single trimer of OmpU (984 pS at +70mV), which corresponds to a conductance of 314 pS. This conductance is equal to about one third of the full conductance of a single trimer of OmpU (984 pS ± 134 pS in 150 mM KCl, pH 7.2), as determined from analysis of trimer insertion events in planar lipid bilayers (Fig. 5). The total current through the patch (level labeled “O”) is also compatible with accommodating three (and no more) such conductance steps. Thus, we will make the initial assumption, based on the typical gating behavior of porins, that the 314 pS step corresponds to the closure of one monomer (but see below). We had reported a 51 pS conductance in a previous publication. This work was done prior to our knowledge of the conductance of trimer insertion events in bilayer. We now believe that the 51-pS steps represent a substate, by analogy with the substate gating that we have documented for OmpF. It is noteworthy that at voltages smaller than the critical voltage for inactivation, a single trimer of OmpU displays only single-step closures. At higher voltages, OmpU will start to inactivate, and, as shown at -170 mV in Figure 1C, the trace will eventually reach a level with a small residual conductance, which comes partly from the leak through the patch and partly from some small leak through the same on each side of the membrane. Fifty micrograms of dip hytanoyl-sn-glycero-3-phosphocholine in pentane (5 mg/mL) were added to both chambers. A lipid bilayer was formed over the hole by lowering and raising the level of buffer in one chamber. Ten ng of pure protein were added to one chamber only (cis-side), from a 2 ng/µL stock solution prepared by dilution of the purified protein in a 1% Octyl-POE solution in buffer A.

Figure 1. Behavior of OmpU and OmpT at neutral pH. (A–C) Representative current traces of a single OmpU trimer activity at the indicated pipette voltages were obtained at pH 7.2. (B) is the expanded version of the 1 sec segment indicated by the asterisk in (A). The zero current level (“zero”) and the fully open state (“O”) are indicated. For traces (A and B), the single observed closed state, referred to in the text as the “single-step closure”, is indicated by “C”. For (C), the three main closed levels are indicated by tick marks. Since other closed levels are observed and there is uncertainty as whether these three main levels do indeed represent the closure of each monomer in sequence, the tick marks are not labeled with numeric C’s. Note that because the trace at -170 mV is so compressed, we are only displaying 1 out every 10 points. (D–F) Representative current traces of a single OmpT trimer activity at the indicated pipette voltages were obtained at pH 7.2. The zero current level (“zero”), the fully open state (“O”) and the levels corresponding to the closure of each monomer (C1 to C3) are indicated. Note the increased spontaneous gating frequency of OmpT relative to OmpU, and the more rapid relaxation of OmpT to the inactivated state at negative voltages (different scale bars for C and F).
the apparent closed state, as described for OmpF. It is a hallmark of porin behavior that this inactivation takes place in three distinct steps, believed to correspond to the successive closures of each monomer. The trace of panel C shows that OmpU indeed displays three major closed steps (indicated by tick marks) which may correspond to each monomer, but additional levels of smaller or larger amplitudes are also observed. This is not an idiosyncrasy of patch-clamp, as similar behaviors are observed in planar lipid bilayers as well. In fact, on several occasions, the voltage-dependent inactivation of a single OmpU trimer occurred in four or more steps. Therefore, at high voltages, OmpU shows a complex kinetic behavior, which includes a multiplicity of substates, and/or possibly cooperativity across monomers. At low voltages, only one closed level is observed (labeled “C” in Fig. 1A and B), and we will refer to transitions to this level as “single-step closures.”

On the other hand, in the case of OmpT, transitions that can be ascribed to closures of single monomers are clearly seen. The rapid kinetic activity distinguishes OmpT from other porins, and spontaneous closures of single monomers are observed even at low voltages (Fig. 1D and E). At higher voltages, inactivation occurs in three well-defined steps that are not quite of the same conductance. For the trace shown in Figure 1F, the conductances were 304, 318 and 367 pS, for the 1st, 2nd and 3rd closure, respectively. Differences in the conductance of each monomer closure have also been observed for OmpF. Inactivation of OmpT is also much faster (note the different scale bars for Fig. 1C and F) and occurs at lower voltages than for OmpU, because of different voltage sensitivities, as documented.

Effect of acidic pH on the kinetics of OmpU and OmpT. Because pH is a well-known environmental signal for bacteria and because of its demonstrated effect on porins from other organisms, we tested the effect of pH on the electrophysiological properties of OmpU and OmpT using the patch-clamp technique. Since it is not practical to exchange the pipette solution, only the buffer outside of the pipette was exchanged, and thus the experiments at pH other than 7.2 were performed in asymmetric conditions, where the pH inside the pipette remained at 7.2, and the pH of the bath solution was as indicated in the figures. The traces of OmpU shown in Figure 2 were obtained from the same patch and reveal the behavior of one single trimeric porin throughout different conditions. At pH 7.2, OmpU displays occasional single-step closures, as described in Figure 1. The amplitude histogram shown to the right of the trace illustrates that most of the current dwells at the fully open level marked “O”. The duration and frequency of the single-step closures are so small that a peak is only barely visible on the amplitude histogram (see Supplementary Fig. 1 for dwell time distributions of the marked open and closed states). When the pH is dropped by bath perfusion of the appropriate buffer, we witness an increase in the frequency, as well as in the dwell time, of the closing events. Therefore, the activity of the channel is modulated towards the closed state. Surprisingly, rather than seeing the appearance of additional current levels corresponding to closures of multiple monomers, we continue to observe only single step transitions between the fully open state and a single main closed state. For the rest of the paper, we will refer to these events as “single-step closures” or “single-step closing events.” The average single-step closure dwell time jumps from 7.8 ± 1.5 msec at pH 7.2, to 100 ± 15 msec at pH 6.2. In addition, the size of the current step during the single-step closing events increases, although the voltage remains at +50mV. For example, at pH 5.7 (Fig. 2C), the current steps for this trace average -25 pA, while they are -16 pA at pH 7.2. The amplitude histograms clearly illustrate the progressive increase in the gap between the two peaks, as the bath pH is made more acidic.

Surprisingly, the increase in the current for the single-step closing events is not associated with an increase in the total conductance of the porin, as indicated by the position of the fully open level (“O”) relative to the zero current level (dashed line). In fact, as can be clearly seen in Figure 2C and D, it is no longer possible to fit three such current steps within the total amount of current flowing through the trimer. In addition, close inspection of the current trace indicates that the closures and re-openings occur in one step, without any short-lived transitions to levels of intermediate conductances. However, inspection of the noise in both the open and the closed states indicates the presence of sub-conductance levels. Thus, it seems that in these conditions, the single-step closing events can no longer be explained by the closure of a single monomer, suggesting that coordinated events must take place between the monomers. At pH 5.2, the porin displays very long closures (average of 4791 ±119 msec), and rarely comes back to an open state. This leads to an inversion in the height of the peaks of the amplitude histogram, which reflect the relative occupancy of the channel in each state. It is important to note that even at pH 5.2, the current level of the closed state is still distant from zero, and in fact from the 2–3 pA current level expected at 50 mV if the trimer was fully inactivated (see Fig. 6 for example). Finally, although the perfusions for this
experiment were performed in the order displayed in Figure 2, we have repeatedly observed these effects on more than 12 patches of OmpU regardless of perfusion order, and the effect of pH is fully reversible. Aguilella and colleagues have reported pronounced asymmetries in the behavior of OmpF in the presence of a pH gradient. For OmpU, the fact that the channel is subjected to a pH gradient does not seem to influence the effect. Indeed, we have performed control experiments in planar lipid bilayers, where pH 5.2 buffer was applied symmetrically on both sides of the membranes. In these conditions, the conductance of the single-step closure averaged $470 \pm 8$ pS ($n = 16$), a value similar to the one obtained from patch-clamp experiments ($472 \pm 16$ pS ($n = 7$)).

As documented in Figure 1 and previously, OmpT has a more active kinetic behavior than OmpU at pH 7.2. Numerous closing events of one or sometimes two monomers can be observed within 30 sec (Fig. 3A). The perfusion of acidic buffers does not have as drastic an effect on OmpT as it does on OmpU. As shown in Figure 3B, the frequency and the dwell time of the closing events increase (the dwell time for the first closed state is $11.4 \pm 1.1$ msec at pH 7.2 and $38.5 \pm 5.1$ msec at pH 5.2; Supplementary Fig. 2 for dwell time distributions), leading to an increase in peak heights in amplitude histograms, and the closures exhibit a minor change in their current value. As for OmpU, the effect of pH on OmpT is reversible.

In order to quantitatively document the different sensitivity of the two porins to acidic pH, we have measured the open probability ($P_o$) of each porin with respect to pH (Fig. 4A). Due to increasing the frequency, the dwell time and the size of the closing events, pH has a strong effect on the open probability of OmpU. At pH 7.2, an OmpU trimer is mostly in the open state, and $P_o$ is close to 1. At pH 4.7, the open probability has dropped by more than 50%. OmpT, on the other hand, despite a high frequency of very short closures, keeps a $P_o$ of 70% or higher, even at pH 4.0. The OmpU data was fitted to equation (1) which assumes a single titratable site and uses the Henderson-Hasselbach equation to assign the fractional composition of protonated and unprotonated channel molecules. In the absence of knowing exactly how many residues participate in the pH-dependent control of $P_o$, the $pK$ obtained from the fit should be considered as an apparent $pK$ ($pK_{app}$), and its value was found to be 4.9 for OmpU. The fit for OmpT was found to be unreliable, and the line drawn in the figure represents a trend line with a $pK_{app}$ of 4.0.
Figure 4B documents that the decrease in Po of OmpU is due to a combination of decreased average dwell time at the fully open level, and increased average dwell time of the single-step closure. For this analysis, the average dwell times were measured for single current steps, such as those illustrated in Figure 1, although we cannot ascertain whether they originate from a single monomer or a combination of substates from different monomers. Still, since the events are so clearly defined and reproducible across patches, we believed that there is validity in treating them as single events for the purpose of documenting the pH-dependence of the measured dwell times. Note the different ordinates for the two plots, and in particular the fact that the average closed times were plotted on a logarithmic scale. Indeed an increase of over two orders of magnitude is observed for the average closed time, while the average open time decreases by about 7-fold, when the pH drops from 7.2 to 4.7. At this point, we do not think that the closed state and the fully open state of the single-step transitions represent a protonated and unprotonated forms of the channel, respectively. If this was the case, the average closed time would represent the residence time of the bound proton(s) and should be independent on proton concentration, which is not the case here. Rather, it appears that the kinetics of gating is modulated by pH, at the level of both the opening and closing rate constants.

Effect of acidic pH on the size of OmpU current steps. A surprising effect of acidic pH on OmpU is the increase in the size of the single-step closing events. In order to obtain the conductance of these transitions at various pHs, we constructed current-voltage relationships (I/V curves) by plotting the single-step closure currents (see Fig. 5A for illustration of the measured currents) over a wide voltage range. Figure 5B shows the averaged data obtained from at least three experiments for each voltage and each condition. Note that in all conditions, the I/V curves go through zero, and show a symmetric behavior in the positive and negative voltage ranges, indicating that proton flow does not contribute to the measured current and the absence of asymmetries due to the proton gradient. At low voltages (between + and -70 mV), the single-step closing events exhibit a voltage independent conductance, as illustrated by the linear relationship between voltage and amplitude. However, the conductance decreases when the voltage gets higher, in the negative or in the positive range, giving rise to a sigmoidal dependence. This non-ohmic behavior at high voltages is observed for every pH. This behavior is not observed when the total current (flowing through a single trimer; see Fig. 5A) is plotted against voltage; here the relationship is linear, as illustrated for a single experiment in Figure 5C. In addition, Figure 5C demonstrates that the total trimeric current remains unchanged when the same patch is subjected to increasingly lower pH's. This difference in the I/V curves of the single-step closing events vs. trimeric conductance highlights the idea that the single-step closing events observed at acidic pH represent some kind of concerted behavior of the subunits within a trimer.

To verify the effect of pH on the trimeric conductance, we conducted planar lipid bilayer experiments, and measured the size of current jumps immediately after addition of purified OmpU to the cis-side of the membrane. It is generally believed that these insertion events represent the conductance increases observed as single or multiple porin trimers spontaneously insert in the bilayer. Figure 5D shows the amplitude histogram built from 142 conductance measurements performed at pH 7.2 (on both sides of the membrane). The first peak, corresponding to the insertion of one single trimer, is fitted to a Gaussian distribution (thick line) with the Igor multi-peak routine, and gives a mean peak value of 984 ± 135 pS. Although we can see the second peak, corresponding to the insertions of two porin trimers, there were not enough events to fit it reliably to a Gaussian plot. A similar experiment was performed at pH 6.2 with 120 events, and the Gaussian fit to the first peak distribution gave a single trimer conductance of 891 ± 133 pS (Fig. 5E). Together with the plot of Figure 5C, these results reinforce the observation that, although acidic pH increases the conductance of the single-step closing events, it introduces only a relatively minor change in the trimeric conductance. We had hoped to carry out the insertion analysis at even lower pHs, but unfortunately, at those pHs, the porin is mostly closed, which makes the detection of insertion events unreliable.

Figure 5F compares the pH dependence of the change in conductance observed for OmpU and OmpT. For each porin, the conductances of the closing events were measured in individual experiments and averaged: for OmpT, we measured the current steps corresponding to the first closure from the fully open state (C1 in Fig. 3); for OmpU, we used the current values corresponding to the single-step closures (as defined in Fig. 5A and as level C in Fig. 2). For OmpT, the conductance was obtained as the slope of the linear I/V plots, which remains constant over the voltage range investigated (data not shown). For OmpU, only the linear part of the I/V plots (-70 to +70 mV) was used. At pHs 4.7 and 4.2, the low open probability of channel made the measurement of the single-step closing event currents challenging. In order to get enough data to calculate a reliable conductance, we had to reopen the channel using high positive voltages, and then immediately bring the patch voltage back to a voltage where the I/V relationship is linear (usually +50 mV). The channel would almost immediately close again at those lower voltages, but we were able to monitor and measure the size of this single-step closing event. Therefore, the conductances obtained at pH 4.7 and 4.2 were not calculated from a full I/V curve, but from single measurements at +50 mV. Figure 5F shows that the conductance for OmpT slightly decreases at acidic pHs, but a much more drastic change is observed for OmpU, where the conductance increases by more than 50%. Because of this observation, we decided to extent the measurements in the alkaline range, and included a value at pH 9.2. Surprisingly, the conductance of the OmpU single-step closures continues to decline beyond neutral pH, while that of OmpT monomers remains relatively unchanged. The whole pH dependences for OmpU and OmpT conductances were fitted to equation (1). The fits returned pK values of 6.1 and 5.1 for OmpU and OmpT, respectively. In each case these values are more basic than for the Po fit, suggesting that a different set of residues control the pH dependence of Po and conductance.

OmpU still shows multi-step closures at low pH. A hallmark of porin behavior is a voltage induced inactivation. When a constant high voltage is applied, porins usually display three-step closures, corresponding to the sequential closure of each monomer, with kinetics that depends on the magnitude of the voltage and on the specific porin. Can this stepwise closure still be observed in OmpU at acidic pHs, and is it compatible with the single-step closing events induced at those pHs? Figure 6 shows two representative experiments at pH 5.2 demonstrating that this is the case. In each experiment, the patch potential was first maintained at -30 mV, to verify that the channel displayed the single-step closing events with large conductance typical of acidic conditions. A segment of the recording at -30 mV is shown in the first part of each trace. Later on
in the experiments, the patch voltage was stepped to -150 mV. Here we witness the sequential multi-step closure with slow kinetics typical of voltage dependent inactivation. Note that the traces are plotted in pS (rather than pA), to ease the comparison in the size of the events at low and high voltages. Several observations are worth noting: (1) during the process of voltage dependent inactivation porins behave “as usual”, i.e., the behavior of presumed individual monomers can be recognized; (2) therefore these patches indeed contained a single fully functional trimer at low pH; (3) the final current value, after inactivation is complete at high voltage, is much smaller than the “closed” level (labeled “C” in Fig. 2) of the single-step closing events seen at lower voltages; (4) therefore, the single-step closures observed at lower voltage do not represent the full cooperative closure of the three monomers. These experiments reveal that two types of closures of OmpU can occur at acidic pH. First the high conductance, single-step closures induced by pH, which match to more than one third of the total conductance, and secondly closures of smaller conductance that can cumulatively drive OmpU to a fully inactivated state. The former type of closures is typically seen at low potentials, where channel inactivation does not occur. The latter type occurs at high membrane potentials. Note that at these voltages, gating to substates also occurs, and occasional large events are also seen (for example, the first closure at -150 mV of Fig. 6B). This coexistence of the two types of closures suggests an interplay between the effect
The effect of protons on channel properties has been documented on both prokaryotic\textsuperscript{16,32,33} and eukaryotic channels.\textsuperscript{34-38} The effects have been varied, ranging from increase in open channel noise in α-hemolysin\textsuperscript{22} and OmpF\textsuperscript{16} to changes in the single channel conductance and substates,\textsuperscript{21,25,37,38} to gating kinetics modification, for example induction of channel closure.\textsuperscript{16,17,19} It is generally accepted that these effects originate from titration of accessible ionizable groups that control ion flow or channel gates. In particular, Bezrukov and colleagues have made a detailed study of the dynamics of protonation from the noise analysis of currents through the Staphylococcus aureus α-toxin, where they estimated the number of ionizable groups to be 4, and obtained the rate constants for the reversible protonation reactions.\textsuperscript{22} Our results indicate that increase in open channel noise is rather a minor aspect in the effect of protons on OmpT and OmpU. The major effect is the promotion of closures that eventually leads to a decreased overall open probability. This is akin to what is observed with OmpF; although the kinetic patterns are quite different. Traces of OmpF activity at low pH show that the channel is readily open upon a voltage step (for example to 100 mV) but then closes rapidly and in three steps.\textsuperscript{16,17} The relaxation time for channel closure is increased as the pH is more acidic (see for example Fig. 1 in ref. 16). This kinetic pattern is similar to the voltage-dependent inactivation, and indeed, it has been suggested that acidic pH decreases the voltage threshold for voltage-dependent gating of OmpF,\textsuperscript{39} thus resulting in fast inactivation at voltages that would not cause inactivation at neutral pH. On the contrary, the increase in closing activity of OmpT or OmpU is visible immediately upon a voltage step. Thus, although inactivation may occur at high enough voltages (Fig. 6), the typical kinetic pattern of OmpU at acidic pH is that of sustained—albeit longer and more frequent—but still reversible transitions between open and closed states. This pattern suggests a pH-dependent modulation of the rate constants for channel opening and closing, rather than the promotion of an inactivated state, as is the case for OmpF.\textsuperscript{17}

The typical behavior of multiple independent channels in response to a closure-inducing modulator is for each to increase their closing frequency and/or closing duration in such a way that when the stimulus is strong enough, multiple closing steps are individually observed and the traces take a “staircase” appearance. An example of this behavior is shown by OmpT at pH 5.2 in Figure 3. The most puzzling observation regarding OmpF modulation by pH is that multiple steps corresponding to the closures of individual monomers are not seen. Rather the pH dependence of the modulation is manifested by a more frequent, longer and larger (in current) single-step closure. Furthermore, the increase in the size of the closing transition is without a concomitant increase in the macroscopic current flowing through the whole trimer, implying that the intrinsic channel conductance has not been changed (as confirmed by the insertion analysis) and that no “loss” of channels (for example a permanent closure of one or two monomers) has occurred. We will offer here some speculative explanations for this peculiar behavior.

The most radical proposal is that, although is has a trimeric composition, OmpU forms a single pore, that closes to various extents depending on the pH. Notwithstanding the case of eukaryotic channels built on α-helical motifs, there is precedence for single β-barrel pores formed by multimeric proteins, for example the outer membrane component of the multidrug resistance pathways TolC\textsuperscript{20} and VceC\textsuperscript{40} and the trimeric autotransporter Hia.\textsuperscript{41} Arguments in

\begin{figure}
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\caption{OmpU can display multiple sequential stepwise closures at acidic pH when induced at high voltages. Representative patch clamp traces of a single OmpU trimer from two separate patches (A and B) at pH 5.2 and at a pipette voltages of -30 mV and -150 mV, as indicated. The traces are shown as conductance changes (in pS) to allow a direct comparison of the size of closing events at the two voltages. The dashed line shows the 0 pS conductance level (there is a slight artefactual offset between the two voltages of trace (A) The tick marks on the right hand side represent the fully open level of a single trimer (marked “O”) and the main conductance levels of the trace at -150 mV, corresponding to the closure of presumed single monomers. Note that for both traces the single monomer conductance changes are smaller than that of the single-step closing events observed at -30 mV.}
\end{figure}
favor of this model are the observation of single-step closures and the apparent continuum in conductance derived from these current steps as the proton concentration increases, which may reflect a progressive electrostatic effect on the channel walls ultimately impacting the amount of current carried by the electrolytes. Arguments against this scenario is that the OmpU amino acid sequence is close enough to that of OmpF that a homology model can be constructed based on the OmpF structure, and this model shows a monomeric structure very similar to that of OmpF. In addition, sequential closures in three steps, a hallmark of trimeric porin activity, is seen at high voltages. But it is worth pointing out that additional steps are also seen on occasions, supporting the idea that the voltage dependent inactivation may involve closing to substates. These substates are not incompatible with a model where a single pore exists, as found in TolC.

Alternatively, we can consider that OmpU is indeed constructed as a typical triple barrel channel. Two scenarios may be envisioned. In the first scenario, the single-step closure corresponds to the highly cooperative closure of all three monomers; the contribution of each monomer to current change during this step is not, however, a third of the total trimeric conductance, but some fraction of it, which becomes increasingly larger as the pH becomes more acidic. In other words, each monomer closes partially, but the fractional closure is dependent and the partial closures of each monomer sum up to the current change observed during the closing step. Arguments in favor of this model are the documented electrostatic effects of protons on channel conductance. However, this model posits a high degree of cooperativity between monomer(s), which has never been documented.

In the second scenario, the single-step closure corresponds to the full closure of one monomer, with concomitant adjustment of the conductance of the other two monomers during the closure, such that the amount of current remaining through these two open monomers decreases progressively as the pH is lowered. Arguments in favor of this model are that it readily explains the observed single-step closure, without the need to invoke gating cooperativity. However, this is a more complicated situation as it would imply that the monomer that closes has a different conductance from that of the other two monomers. In both of these scenarios, the apparent "closure" may not represent an actual structural event, but the fractional closure is dependent and the partial closures of each monomer sum up to the current change observed during the closing step. Arguments in favor of this model are the documented electrostatic effects of protons on channel conductance. However, this model posits a high degree of cooperativity between monomer(s), which has never been documented.

**References**

1. Krukonis ES, DiRita VJ. From motility to virulence: Sensing and responding to environmental signals in Vibrio cholerae. Curr Opin Microbiol 2003; 6:186-90.
2. Dhillon J, Klose KE. Vibrio cholerae and cholera: Out of the water and into the host. FEMS Microbiol Rev 2002; 26:125-39.
3. Skorupski K, Taylor RK. Control of the ToxR virulence regulon in Vibrio cholerae by environmental stimuli. Mol Microbiol 1997; 25:1003-9.
4. Gupta S, Chowdhury R. Bile affects production of virulence factors and motility of Vibrio cholerae. Infect Immun 1997; 65:1131-4.
5. Hsung DT, Melkonian J. Bile acids induce cholera toxin expression in Vibrio cholerae in a ToxT-independent manner. Proc Natl Acad Sci USA 2005; 102:9328-33.
6. Crawford JA, Kaper JB, DiRita VJ. Analysis of ToxR-dependent transcription activation of ompU, the gene encoding a major envelope protein in Vibrio cholerae. Mol Microbiol 1998; 29:235-46.
7. Li CC, Crawford JA, DiRita VJ, Kaper JB. Molecular cloning and transcriptional regulation of ompT, a ToxR-regulated gene in Vibrio cholerae. Mol Microbiol 2000; 35:189-203.
8. Provenzano D, Lauriano CM, Klose KE. Characterization of the role of the ToxR-modulated outer membrane porins OmpU and OmpT in Vibrio cholerae virulence. J Bacteriol 2001; 183:3652-62.
9. Duret G, Delcour AH. Deoxylcholic acid blocks Vibrio cholerae OmpT but not OmpU porin. J Biol Chem 2006; 281:19899-905.
10. Nikaido H. Molecular basis of bacterial outer membrane permeability revisited. Microbiol Mol Biol Rev 2003; 67:593-656.
11. Chakrabarti SR, Chaudhuri K, Sen K. Das J. Porins of Vibrio cholerae: Purification and characterization of OmpU. J Bacteriol 1996; 178:524-30.
12. Wiibbenmeyer JA, Provenzano D, Landry CE, Klose KE, Delcour AH. Vibrio cholerae OmpU and OmpT porins are differentially affected by bile. Infect Immun 2002; 70:121-6.
13. Simonet VC, Baue A, Klose KE, Delcour AH. The Vibrio cholerae porins OmpU and OmpT have distinct channel properties. J Biol Chem 2003; 278:17539-45.
14. Subbarao GY, van den Berg B. Crystal structure of the monomeric porin OmpG. J Mol Biol 2006; 360:750-9.
15. Fourel D, Bernard A, Pages JM. Involvement of extruded polyepideptide loops in trimeric stability and membrane insertion of Escherichia coli OmpF porin. Eur J Biochem 1994; 222:625-30.
16. Nesterovitch EM, Rostovtseva TK, Bezrukov SM. Residue ionization and ion transport through OmpF channels. Biophys J 2003; 85:3718-29.
17. Badal A, Quurb R, Mehrzain M, Wiibbenmeyer J, Delcour AH. Deletions of single extracellular loops affect pH-sensitivity, but not voltage-dependence, of the E. coli OmpF Protein Eng Des Sel 2004; 17:665-72.
18. Liu N, Delcour AH. Inhibitory effect of acidic pH on OmpC porin: Wild-type and mutant studies. FEBBS Lett 1998; 436:160-4.
19. Conlan S, Bayley H. Folding of a monomeric porin, OmpG, in detergent solution. Biochemistry 2003; 42:9453-65.
20. Koronakis V, Sharff A, Koronakis E, Luisi B, Hughes C. Crystal structure of the bacterial membrane protein ToxC central to multidrug efflux and protein export. Nature 2000; 405:914-8.
21. Andersen C, Hughes C, Koronakis V. Electrophysiological behavior of the ToxC channel-tunnel in planar lipid bilayers. J Membr Biol 2002; 185:83-92.
22. Kasianowicz JJ, Bezrukov SM. Protonation dynamics of the alpha-toxin ion channel from spectral analysis of pH-dependent current fluctuations. Biophys J 1995; 69:94-105.
23. Benz R, Janko K, Lauger P. Ionic selectivity of pores formed by the matrix protein (porin) of Escherichia coli. Biophys Acta 1979; 551:238-47.
24. Bucheler LK, Kuwamoto S, Zhang H, Rosenbusch JP. Plasticity of Escherichia coli porin channels: Dependence of their conductance on strain and lipid environment. J Biol Chem 1991; 266:24446-50.
25. Todt JC, Rocque WJ, McGoarty EJ. Effects of pH on bacterial porin function. Biochemistry 1992; 31:10471-8.
26. Delcour AH, Martina B, Adler J, Kiang C. Voltage-sensitive ion channel of Escherichia coli. J Membr Biol 1989; 112:267-75.
27. Badal A, Iyer R, Delcour AH. Subconductance states in OmpF gating. Biochim Biophys Acta 2004; 1664:100-7.
28. Danelon C, Suenga A, Winterhalter M, Yamato I. Molecular origin of the cation selectivity in OmpF porin: Single channel conductances vs. free energy calculation. Biophys Chem 2003; 104:591-603.
29. Rostovtseva TK, Nesterovitch EM, Bezrukov SM. Partitioning of differently sized poly(ethylene glycol)s into OmpF porin. Biophys J 2002; 82:160-9.
30. Alcaraz A, Ramirez P, Garcia-Gimenez E, Lopez ML, Andrio A, Aguilella VM. A pH-tunable nanofluidic diode: Electrochemical rectification in a reconstituted single ion channel. J Phys Chem B Condens Matter Mater Surf Interfaces Biophys Chem 2006; 110:21205-9.
34. Hebert SC, Desir G, Giebisch G, Wang W. Molecular diversity and regulation of renal potassium channels. Physiol Rev 2005; 85:319-71.
35. Wemmie JA, Price MP, Welsh MJ. Acid-sensing ion channels: Advances, questions and therapeutic opportunities. Trends Neurosci 2006; 29:578-86.
36. Zhang X, Zeng X, Lingle CJ. Slo3 K+ channels: Voltage and pH dependence of macroscopic currents. J Gen Physiol 2006; 128:317-36.
37. Root MJ, MacKinnon R. Two identical noninteracting sites in an ion channel revealed by proton transfer. Science 1994; 265:1852-6.
38. Pietrobon D, Prod’hom B, Hess P. Interactions of protons with single open L-type calcium channels: pH dependence of proton-induced current fluctuations with Ca2+, K+, and Na+ as permeant ions. J Gen Physiol 1989; 94:1-21.
39. Saint N, Lou KL, Widmer C, Luckey M, Schirmer T, Rosenbusch JP. Structural and functional characterization of OmpF porin mutants selected for larger pore size. II. Functional characterization. J Biol Chem 1996; 271:20676-80.
40. Federici L, Du D, Walas F, Matsumura H, Fernandez-Recio J, McKeegan KS, Borges-Walmsley MJ, Luisi BF, Walmsley AR. The crystal structure of the outer membrane protein VceC from the bacterial pathogen Vibrio cholerae at 1.8 Å resolution. J Biol Chem 2005; 280:15307-14.
41. Meng G, Surana NK, St Geine IIIrd JW, Waksman G. Structure of the outer membrane translocator domain of the Haemophilus influenzae Hia trimeric autotransporter. Embo J 2006; 25:2297-304.
42. Gurnev PA, Oppenheim AB, Winterhalter M, Berezuk SM. Docking of a single phage lambda to its membrane receptor maltoporin as a time-resolved event. J Mol Biol 2006; 359:1447-55.