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Determinants of the Proton Selectivity of the Colicin A Channel

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ABSTRACT: The channel formed by colicin A in planar lipid bilayers has an outsized selectivity for protons compared to any other ion, even though it allows large ions, such as tetraethylammonium, to permeate readily. A mechanism to account for this discrepancy remains obscure. We considered that protons may traverse a separate pathway but were unable to find any evidence for one. Manipulations that interfere with ionic conduction, such as replacing some of the water in the pore with a nonelectrolyte, reduce the proton current along with the ionic current. Lipids have been proposed to play a structural role in the channel, but we found that the proton selectivity was unaffected by various gross changes in the lipid composition of the bilayer, effectively ruling out any specific effect of lipids in the selectivity and offering no support for their role in structure. The 10-helix channel-forming domains of colicins Ia and E1 are structurally homologous to that of colicin A but do not select so remarkably for protons; thus we were able to use them to probe for the regions responsible for the high selectivity. Using hybrids made by helix swapping among these proteins, we found that the anomalous selectivity could be localized to the five C-terminal helices of colicin A.

In a recent paper (*1*), we showed that the channel formed by colicin A has an anomalously high proton selectivity, even though the channel also conducts large ions. In this paper we attempt to examine certain characteristics of the colicin channel that might be related to this trait. In addition, we consider the reverse question: what can this feature reveal about the structure of the colicin A channel?

Ion channels will generally conduct protons along with whatever ions fulfill their biological role, sometimes with surprisingly high selectivity. For example, the V-dependent sodium channel selects for protons over sodium by a factor of over 200 (*2*). This is often of little consequence since the concentration of protons is usually quite low and biological fluids are generally well buffered. A subset of channels, where proton conductance has a biological role, has much higher, even essentially perfect, selectivity for protons over other ions (*3*). Both types of channels are thought to (or, in some cases, known to) have selectivity filters of atomic dimensions. Colicin A is a rare exception that lacks such a filter yet selects for protons better than any known channels other than dedicated proton channels. It has been reported that the capsaicin receptor TRPV1 is large enough to transport TEA[1] and other large cations, yet exhibits a P_{H^+}/P_{Cs^+} of over 1000 under some conditions (*4*). This channel has also been reported to exhibit time-dependent changes in permeability, as if it were increasing its diameter with time (*5*), which suggests a possible explanation for the reported discrepancy between proton selectivity and lumen size; i.e., the high proton selectivity and the permeability to large ions are not attributes of the same channel.

The central conundrum—how can a large pore select so effectively for protons—would be obviated if the proton current followed a pathway outside the principal pore. Therefore, we looked for agents that might modulate only one of these two putatively separate currents. For example, we modified and removed the three histidine residues in the channel-forming domain. Histidines are known to modulate proton current in some proteins (*6*−*9*), and the presence of three in colicin A offered targets that might be involved in a separate proton pathway. Likewise, we considered that a putative proton pathway might involve membrane lipids. The colicin channel has been hypothesized to incorporate lipids into its structure (*10*, *11*). The details of such a structure are purely speculative, but it is reasonable to assume that any arrangement would be sensitive to the nature of the lipids involved. We thus measured colicin proton selectivity in a variety of lipids.

Apart from the question of its mechanism, the high proton selectivity of colicin A provides a new tool to investigate the structure of the channel. The channel-forming domain, which forms a bundle of 10 α-helices in solution, is thought to arrange itself into at most four transmembrane segments in order to form a channel in a lipid bilayer (*12*−*14*). It is not known if all four segments are directly involved in forming the pore or the putative separate proton pathway. The role of helix 1 (the most upstream helix) is particularly problematic, since it has been shown by several techniques to be unnecessary for channel formation (*15*−*18*). We used proton selectivity to probe the role of the upstream and downstream regions of the channel-forming domain in the channel structure in two ways: We made hybrid colicins from colicin A and colicin Ia or E1, which display markedly different proton selectivities, and we looked at the effect of trypsin applied to the trans side of the membrane in the presence of open channels, which converts them into a truncated form with different conduction properties.

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[1]Abbreviations: DEPC, diethyl pyrocarbonate; GMO, glycerol monoolein; PC, phosphatidylcholine; PI, phosphatidylinositol; PNP, Poisson−Nernst−Planck; TEA, tetraethylammonium; V_r, reversal potential.

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MATERIALS AND METHODS

Planar Bilayer Experiments. Folded planar bilayer membranes were formed, and reversal potential was determined as previously described (*1*, *12*). The most accurate and reproducible measurements are made using the tail current method, which isolates the set of channels that gate (i.e., open or close at a measurable rate) at the reversal potential. Some of the constructs discussed below gate poorly at accessible reversal potentials, and for those the reversal potential can only be determined by zeroing the current in an ion (proton, in this case) gradient.

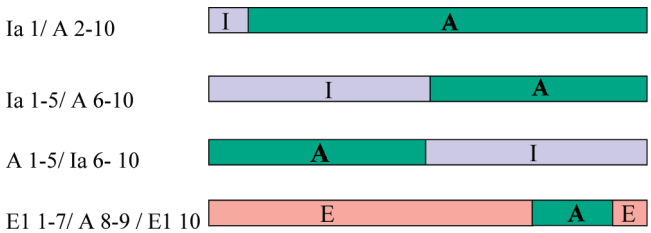
Modification of Histidines in Colicin A. The procedure of Miles (*19*) was used. One milliliter of wt colicin A in 0.02 M phosphate buffer, pH 6.5, at an initial OD_{240} of 1.9 was transferred to a quartz cuvette. One microliter of fresh 100 mM diethyl pyrocarbonate in ethanol was added and the reaction followed by monitoring the absorption at 240 nm. After 20 min the OD_{240} had increased by 0.262, and the reaction was stopped with 1 μL of 1 M imidazole.

Protein Expression and Mutagenesis. Whole wild-type colicin A was expressed in *Escherichia coli* as previously described (*20*). Hybrid colicins were constructed from plasmids pLR1, which encodes wt colicin A, pColE1, which encodes wt colicin E1, and pKS-Ia, which encodes wt colicin Ia. DNA fragments encoding colicin A, E1, and Ia C-terminal domains were amplified from these by PCR using appropriate 5′ and 3′ primers. The 5′ primers encoded a 6His tag and contained the *Eco*RI site at their N-terminus. The 3′ primers contained the *Hin*dIII site at their N-terminus. The resulting DNA fragments were cut by *Eco*RI and *Hin*dIII and ligated into the pJF_{35}-PhoA cut with the same enzymes. The resulting plasmids (p6HisCT-A, p6HisCT-E1, and p6HisCT-Ia) encode 6HisCT-A, 6HisCT-E1, and 6HisCT-Ia, respectively. The DNA fragment encoding the first seven α-helices of CT-E1 (codons 337−475) was amplified by PCR with appropriate 5′ and 3′ primers. The DNA fragment encoding α-helices 8 and 9 of CT-A (codons 534−577) was amplified by PCR with appropriate 5′ and 3′ primers. The 3′ primer also encoded α-helix 10 of pfColE1 (codons 515−522). The resulting PCR fragments were assembled by overlap PCR. This generated the CT-E1AE1 gene, which was inserted into the *Eco*RI and *Hin*dIII sites of pJF_{35}-PhoA. The DNA fragment encoding the first helix of CT-Ia (codons 435−468) was amplified by PCR with appropriate 5′ and 3′ primers. The DNA fragment encoding α-helices 2−10 of CT-A (codons 420−592) was amplified by PCR with appropriate 5′ and 3′ primers. The resulting PCR fragments were assembled by overlap PCR. This generated the CT-Ia 1/A 2−10 gene, which was inserted into the *Eco*RI and *Hin*dIII sites of pJF_{35}-PhoA. The DNA fragment encoding the first five α-helices of CT-Ia (codons 435−542) was amplified by PCR with appropriate 5′ and 3′ primers. The DNA fragment encoding α-helices 6−10 of CT-A (codons 494−592) was amplified by PCR with appropriate 5′ and 3′ primers. The resulting PCR fragments were assembled by overlap PCR. This generated the CT-Ia 1−5/A 6−10 gene, which was inserted into the *Eco*RI and *Hin*dIII sites of pJF_{35}-PhoA. Finally, the DNA fragment encoding the first five α-helices of CT-A (codons 389−490) was amplified by PCR with appropriate 5′ and 3′ primers. The DNA fragment encoding α-helices 6−10 of CT-Ia (codons 536−626) was amplified by PCR with appropriate 5′ and 3′ primers. The resulting PCR fragments were assembled by overlap PCR. This generated the CT-A 1−5/Ia 6−10 gene, which was inserted into the *Eco*RI and *Hin*dIII sites of pJF_{35}-PhoA. The genes coding for all of the mutant proteins were placed under tac promoter control. The proteins were then expressed with standard methods and purified on a nickel column.

Table 1: Reversal Potentials of Colicin Hybrids[a]

	fast gating	slow gating
Ia wt	−3 (n = 10)	
A wt	−21 (n = 10)	−15 ± 2 (n = 6)
wt CT, col A		−13.6 ± 0.6 (n = 4)
wt CT, col Ia		−1 ± 2 (n = 6)
CT-Ia 1/A 2−10	−25 (n = 1)	−11 (n = 2)
CT-Ia 1−5/A 6−10	−26 (n = 2)	−13.1 ± 1.7 (n = 3)
CT-A 1−5/Ia 6−10		−1 ± 1 (n = 4)
wt E1	−3.5 (n = 2)	
wt E1[b]	+14 ± 0.3 (n = 4)	
E1 1−7/A 8−9/E 1−10[b]	+24 ± 2 (n = 3)	
A wt[b]	+34 ± 2 (n = 4)	

[a]The pH gradient is 5/8 except where indicated. pH 5/8 experiments were performed with asolectin membranes in 0.1 M KCl, 5 mM $CaCl_2$, and 1 mM EDTA with 20 mM malate, pH 5.0, on the cis side and 20 mM HEPES, pH 8.0. pH 5/3 experiments have 5 mM malic acid on both sides. [b]pH 5/3 gradient.



Single amino acid mutations were made using the Stratagene QuickChange kit.

PNP software was kindly provided by T. Bargiello, Albert Einstein College of Medicine.

RESULTS

Hybrid and Truncated Colicins. We constructed four hybrid colicins as described in Materials and Methods. All four were designed to be "helix swaps"; i.e., some helices (identified from the X-ray structures) from one colicin were replaced with homologous helices from another. Since the hybrids were made in isolated, channel-forming C-terminal domain constructs, "wt" CT col A and "wt" CT col Ia are included in Table 1 for comparison.

Like whole colicin A (*20*), the Ia/A hybrids appear to have both fast and slow gating states, and we have reported separate values of V_r for these states where possible, although we have not explicitly studied the gating here. Channels formed from the hybrid C-terminal domains gated poorly at the small voltages of these reversal potential experiments, making the determination of V_r by tail currents problematical. As seen in Table 1, the two hybrids that retain the C-terminal five helices of colicin A behave like whole colicin A in this experiment. The A/Ia construct, which has the C-terminal five helices of colicin Ia in the colicin A background, behaves like wt colicin Ia; that is, it lacks colicin A's inflated proton selectivity. The fourth hybrid is essentially colicin E1 with the hydrophobic domain of colicin A (helices 8 and 9) swapped into it in place of its own hydrophobic domain. To analyze this swap, we used a pH 5/3 gradient, which results in a larger absolute value of V_r than does a pH 5/8 gradient.

The colA/colE1 hybrid has a V_r between that of its parent colicins, which suggests that the hydrophobic segment (H8 and H9) is involved in the proton selectivity and also in the overall conductance of the channel if there is no separate proton pathway.

Effect of Trans Trypsin. Colicin Ia channels can be converted from the four-transmembrane wt form into a three-transmembrane form by the action of trypsin on the trans side in the open state (*13*). In order to compare the proton selectivity of the two forms of the channel, we first measured its selectivity in a pH gradient and in low salt (to boost the V_r of colicin Ia into a more easily measured range) and then exposed the open channel to trans trypsin and remeasured the selectivity in the same membrane (Figure 1). In the figure, the reversal potential in a pH 5/8 gradient in 0.015 M KCl was measured to be −10 mV using the tail current technique (shown in expanded form in Figure 1B). Exposure to trans trypsin causes the total current to decrease by a factor of about 6 (Figure 1A), which reflects the conversion of the channels from the wt 50 pS channel to the three-transmembrane segment 7 pS channel (these conductances are representative values in 1 M KCl) (*13*). When the current leveled off to its new value, we determined the selectivity again and found it to be about −17 mV (shown in expanded form in Figure 1C,D). Using a simple model where the pathway for protons is independent of the remainder of the conductance, this change in V_r represents an approximate doubling of P_H/P_K ratio.

In the interest of completeness, we did a similar experiment with colicin A (not shown) and got a sensible result: i.e., trans trypsin does not alter P_H/P_K. This was expected, since trans trypsin has only a small effect on the conductance of open colicin A channels.

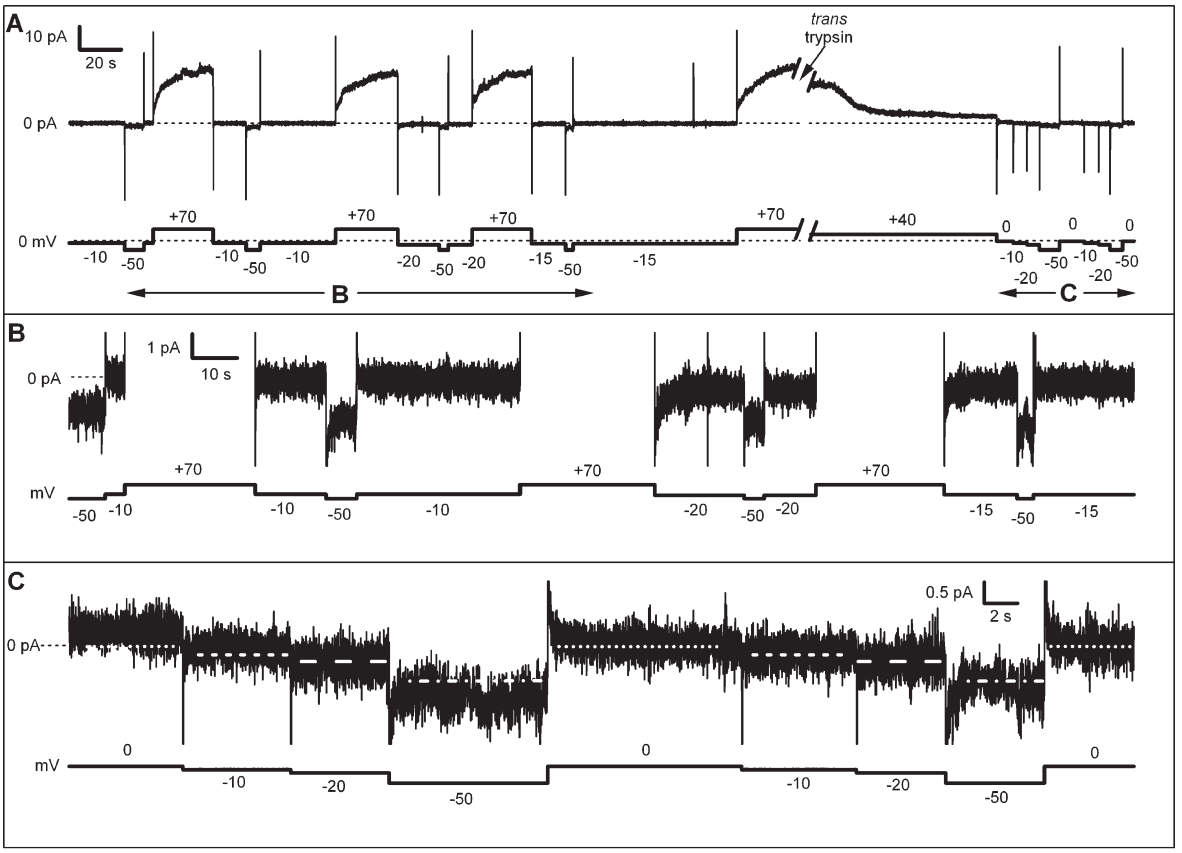
Lipids. The membranes used for most of these experiments were made from acetone-extracted asolectin, which is a somewhat ill-defined mixture of soybean lipids (*21*). The character of the membrane lipid is known to affect colicin channels, and it has long been suspected that lipid molecules may play a direct role in channel structure. We therefore tried to determine whether the membrane lipid played a direct role in the proton conductance. We measured the reversal potential of colicin A in a membrane made from a mixture of two purified lipids designed to have about the same surface charge as asolectin: diphytanoyl PC/PI 4/1. The reversal potential (−21 mV; see Table 2) was essentially identical to what we found for asolectin under the same conditions. When we eliminated the PI from the mixture, thereby eliminating the surface charge (and presumably raising the surface pH), we measured a lower value than we found for asolectin under the same conditions (pH 5/3, 0.1 M KCl) but did not eliminate the anomalous proton conductance (+20 mV vs +34 ± 2 mV (n = 4)). PC membranes do not have a negative surface charge, but the headgroup is not strictly neutral: it is zwitterionic. To see if this could be a factor, we looked at colicin A in membranes made from 100% glycerol monooleate, which is not a phospholipid, having neither a phosphate nor a compensating charged headgroup. Here we found that the reversal potential was slightly larger than what we measured in asolectin under comparable conditions, thus ruling out the phosphate group as a necessity. We also looked at the effect of sterols (which are absent from asolectin, as we prepare it), since sterols have been shown to have an effect on proton conductance in lipid bilayers (*22*); 17% ergosterol had no effect (Table 2). Finally, we looked at colicin A channels in asolectin films painted from decane and hexadecane solutions, in contrast to the films made on squalene-pretreated partitions that are the standard for colicin research. These hydrocarbon solvents are not entirely absent from the bilayer, thickening it slightly in the order decane > hexadecane > squalene. Colicin A is barely active in decane films (presumably because of their thickness), but we nevertheless were able to measure a typical reversal potential (−21 mV) in a pH 5/8 gradient. Its activity in asolectin/hexadecane films is comparable to that in asolectin/squalene films and so is the proton selectivity (−24 mV). These results rule out the squalene or any of its potential contaminants as the cause of the anomaly.

Role of Histidine and Miscellaneous Reagents. The colicin A channel-forming domain contains three histidines, an amino acid that plays an important role in some proton channels (*3*). To investigate whether it is important for the proton conductance of colicin A, we reacted the protein with DEPC, which effectively removes the titratable proton of the imidazole group. We measured a V_r of 28 mV for the reacted protein in a pH 5/3 gradient, somewhat less than for wt under the same conditions (34 ± 2 mV). Further evidence discounting the histidines as major effectors of the proton current comes from the hybrid experiments reported above. The hybrid colicin Ia 1−5/A 6−10 lacks the two upstream histidines found in CT-colicin A, so the result in Table 1 effectively absolves these two of responsibility for the anomalous proton conductance. In order to examine the role of the downstream-most histidine, that is, the C-terminal residue, we changed it to a cysteine and looked at the reversal potential in a pH 5/8 gradient. We measured a value of −18 ± 1 (n = 3) mV for the voltage-dependent conductance and −13 mV for the voltage-independent conductance. Both values are somewhat lower than the corresponding values we found for wt colicin A in similar conditions (−21 and −15 mV).

We tried various reagents in an attempt to decouple the proton conductance from other ions. For example, the divalent cations Cu^{2+} and Zn^{2+} had moderate effects on both the ionic current and the proton selectivity. As such, they were not useful for isolating only one pathway. Amantadine blocks the M2 proton channel (*23*), so we looked at its effect in our system. Neither symmetric 100 μM amantadine nor 500 μM cis amantadine had an effect on the colicin A reversal potential in a pH 5/8 gradient.

Nonelectrolyte Block. Small nonelectrolytes can enter the colicin channel and can, at high concentration, reduce its conductance (*24*). If protons share the same pathway through the channel taken by the major current-carrying ions, then nonelectrolytes should reduce all currents proportionately, with no effect on V_r in pH gradients. If, on the other hand, protons follow a separate pathway, then reducing the ionic current should increase V_r proportionately. We measured V_r of a colicin A-containing membrane in a pH 5/8 gradient using tail currents and then stirred in ethylene glycol to a final concentration of 30% (a small nonelectrolyte that enters the pore and reduces the conductance proportionately) on both sides and repeated the measurement. In this set of experiments V_r was effectively unchanged (−20 mV before ethylene glycol, −21 mV after). Since a credible result depends on effective stirring, we also measured V_r of colicin A in a pH 5/8 gradient in buffers containing 30% ethylene glycol, an experiment that does not depend on stirring. In no case did we measure a V_r more negative than −21 mV.

Permeation Simulation. We used the Poisson−Nernst−Planck (PNP) permeation model to simulate ion currents through the colicin A channel (*25*). In all simulations three ions were present, K^+, Cl^-, and H^+. The model assumes a cylindrical pore. The pore length was fixed as 30 Å, but various diameters were



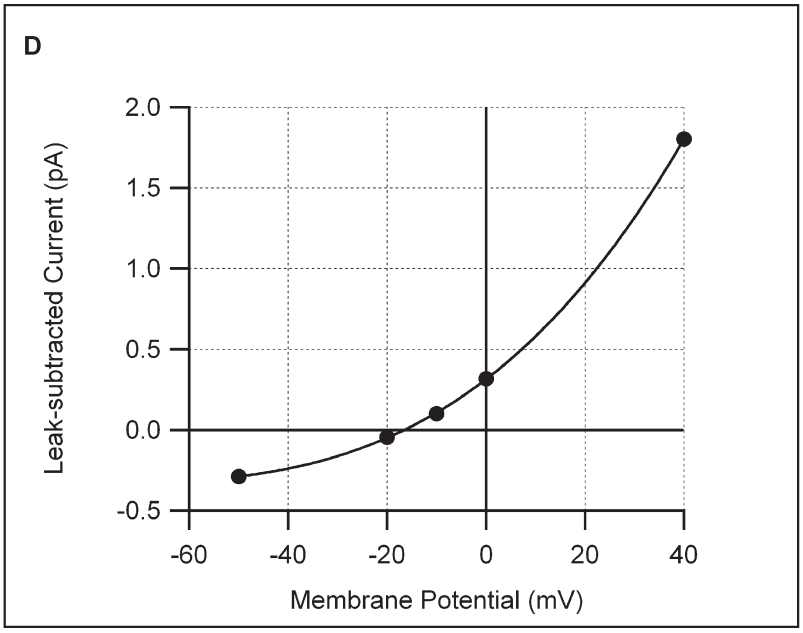


FIGURE 1: Effect of trans trypsin on the H^+ selectivity of colicin Ia. In this experiment, colicin Ia was added to the cis compartment before the section of the record shown. Cis solution: 15 mM KCl, 20 mM malate, pH 5.0. Trans solution: 15 mM KCl, 20 mM HEPES, pH 8.0. (A) A macroscopic current is turned on and off with pulses of +70 and −50 mV, respectively. Pulses to small voltages in the early part of the record are used to measure the reversal potential by examining the direction of the tail currents as the current turns off. Later in the record, the channels are opened at +70 mV, and trypsin is added to the trans compartment. Trypsin is allowed to act on the colicin while the channels are kept open at +40 mV. This causes a reduction in the total open channel current of 5.8-fold in this experiment. Afterward, the reversal potential of the remaining current is determined. The sections of the record labeled B and C are shown in expanded form in panels B and C. (B) V_r is approximately −10 mV. Note that at −15 mV the conductance is "upward" whereas at −10 mV the conductance is almost level, perhaps slightly trending "downward". (C) After trans trypsin little conductance remains and gating is poor. The dotted line formed from small dots at the beginning of this segment of the record represents zero current. Note that the current here is still positive and reverses at about −10 mV. The small dotted line persists across the figure as zero current. The dashed and dotted lines are keyed to voltage and persist across the figure. At this low current level, small leak currents may obscure a voltage-gated current with a higher V_r. To test for this under these conditions, we closed the voltage dependence conductance at −50 mV and subtracted the remaining current (defined as leak) from the total current at each voltage and constructed an I/V curve of the gated current (shown in panel D). (D) I/V curve of the voltage-gated current after trypsin. V_r is approximately −17 mV.

Table 2: Colicin A Reversal Potential (mV) in Different Lipids[a]

Aso	pH 5/8	−21.2 ± 1.3 (n = 10)
Aso	pH 5/3	+34 ± 2 (n = 4)
Diphyt PC/PI 4/1	pH 5/8	−21 (n = 1)
Diphyt PC	pH 5/3	+20 (n = 1)
GMO	pH 5/8	−26 ± 1 (n = 5)
Aso/ergosterol 5/1	pH 5/8	−21 ± 0.5 (n = 3)
Aso in decane	pH 5/8	−21 (n = 1)
Aso in hexadecane	pH 5/8	−24 ± 1 (n = 3)

[a]Symmetric 0.1 M KCl. The solutions also contained 5 mM $CaCl_2$, 1 mM EDTA, and 20 mM malate, pH 5.0, or 20 mM HEPES, pH 8.0, or 20 mM glyceric acid, pH 3.0. V_r was measured using tail currents.

used. The diffusion coefficients and "excess chemical potential" are free parameters that can be fit to the data. Figure 2 shows the results of four such simulations using a pore radius of 10 Å and four different salt and pH conditions. We attempted to replicate the measured reversal potential in a pH 5/3 gradient in symmetric 100 mM KCl (~34 mV, Table 2) and in a pH 3.2/4.6 gradient in 9 mM KCl (~−65 mV (*1*)) (Figure 2A,B), while retaining the experimental values of the conductance (~4 pS) in 1 M KCl (Figure 2C) and the cation selectivity (~32 mV) in a 0.1 M/1 M KCl gradient (Figure 2D). Our best model (which may not be ideal) was able to approximate the measured V_r's of the pH gradients (34 and −69 mV) and the single channel conductance (4 pS), but the V_r in the salt gradient was only 19 mV, rather than 34 mV. However, this was achieved only by assigning a diffusion coefficient to the salt ions in the pore of 2×10^{-8} cm^2/s, which is about 3 orders of magnitude lower than in bulk solution.

DISCUSSION

One Pathway or Two? The high proton selectivity implied by our results could arise from a discrete proton pathway unconnected to the normal ion pathway. The existence of such a pathway would allow for the large luminal diameter deduced for the ion pathway without the need to attribute the anomalous proton selectivity to it alone. If these putative separate pathways were independent, then the measured reversal potential would be the weighted average (weighted by the amount of current each pathway carries) of the reversal potential of each pathway, which would require a proton conductance for colicin A of about 0.2 pS in a pH 5/8 gradient, assuming a conductance in 0.1 M KCl of about 2 pS (representing a current of about 0.004 pA at the reversal potential). The diffusion-limited current for an aqueous ion i of diffusion coefficient D_i is $2\pi FrD_i[i]$, where F is the Faraday constant and r is the capture radius ($r_{pore} - r_{ion}$) (*26*, *27*). Using values for gramicidin (*28*), this works out to about 0.005 pA for protons at pH 5, which can just barely account for the inferred proton current at V_r. Of course, colicin presumably has a larger capture radius than gramicidin. Thus a separate proton pathway cannot be ruled out on these grounds.

The best test for the existence of a separate proton pathway would be to find a blocker for the proton pathway that left the ionic current unaffected, thus shifting V_r to zero in a pH gradient. In this cause we tested amantadine (which blocks M2 proton channels) to no effect. We also looked at several divalent cations (not shown). These reduced V_r somewhat in some experiments, but they also affected the ionic currents and thus provided no support for the dual pathway model. A monovalent ion, NH_4^+, eliminated the high proton selectivity, albeit at a concentration of 30 mM; however, there is good evidence that NH_4^+ interacts with K^+ and H^+ in the

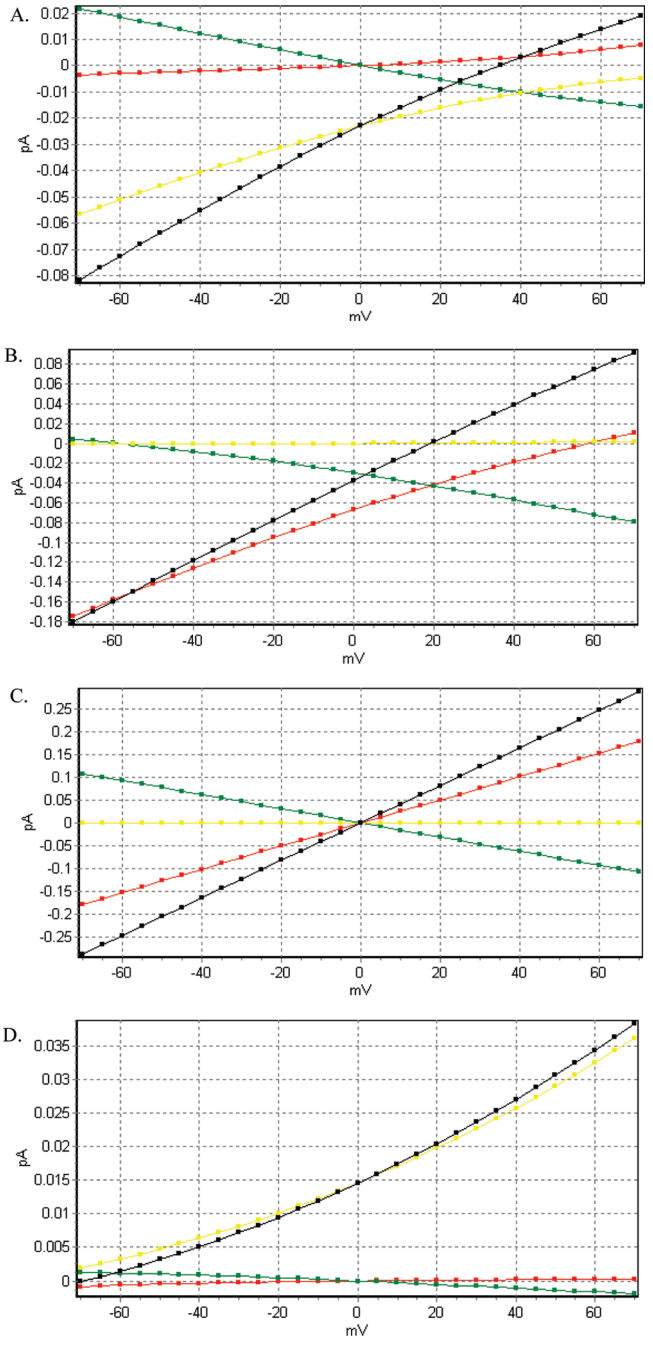


FIGURE 2: PNP simulation of permeation in the colicin A channel. In the model illustrated the radius of the pore was taken to be 10 Å and the length 30 Å. One unit of negative charge was distributed over the central 80% of the channel. Protons were made to experience an excess chemical potential of −20 mV near the center of the pore. Diffusion coefficients were 2×10^{-8} cm^2/s for K^+ and Cl^- and 10^{-5} cm^2/s for H^+. Key: red, K^+ current; green, Cl^- current; yellow, H^+ current; black, total current. (A) pH 5/3, 0.1 M KCl, V_r = 34 mV. (B) pH 5/5, 0.1 M/1 M KCl, V_r = 19 mV. (C) pH 5/5, 1 M/1 M KCl, V_r = 0, g = 4 pS. (D) pH 3.2/4.6, 9 mM/9 mM KCl, V_r = −69 mV.

conductance pathway, so its effect on V_r is not evidence for a separate proton pathway (*1*).

The complementary experiment, blocking the ionic current and looking for an increase of V_r in a pH gradient, suffers from the conundrum that there are no known blockers of colicin channels. It has been reported, however, that certain nonelectrolytes act as partial blockers to the extent that they can fill the pore, such as ethylene glycol (*24*). This small molecule easily enters the pore and reduces the conductance, but it would not be

expected to enter the putative proton pathway, and thus its presence should increase (the absolute value of) V_r proportionately. In the event, it increased V_r by at most 1 mV, far less than predicted by the dual pathway model (~10 mV) for the conditions of the experiment. Thus we can find no evidence for a separate proton pathway.

Permeation through a Single Pathway. If we are to take seriously the idea of a single pathway, we should consider the quantitative implications of the data. A central point of this paper (and ref *1*) is that the observed proton selectivity is difficult to reconcile with the channel's other properties. This statement can be made more precise by the use of a plausible mathematical model of permeation. We used the PNP formulation of Eisenberg, Chen, and their colleagues, an electrodiffusion model that treats the ions as charge distributions in the pore and does not explicitly consider ionic radius. There are, of course, other models of permeation that might be considered, but our aim here is only to gauge roughly the deviation from bulk properties imposed by the channel on the permeant ions in order to substantiate the contention that a 10 Å lumen poses a conceptual problem here, a task that should not depend on the details of the model. First, we ran a simulation of a 10 Å diameter channel in symmetric 1 M KCl at pH 7 using the bulk diffusion coefficients for K^+ and Cl^-. This returns a value of the conductance more than 2 orders of magnitude higher than the measured value (5 pS at pH 5), supporting our suspicion that the movement of ions in the pore is highly restricted compared to bulk water. Retaining the value of 10 Å for the channel diameter, we were able to reproduce the experimental results for the reversal potential in a pH gradient (software considerations limited us to pHs >3) while at the same time reproducing the correct conductance and getting within 30% of the measured value of the cation selectivity in KCl (Figure 2). However, in order to do so, we had to assign diffusion coefficients to the salt ions of $D_{K^+} = D_{Cl^-} = 2 \times 10^{-8}$ cm^2/s, which is 3 orders of magnitude less than their bulk values of 1.9×10^{-5} cm^2/s, along with a value of $D_{H^+} = 10^{-5}$ cm^2/s for protons, which is 1 order of magnitude below the bulk value. In addition, the model assigns an excess chemical potential of −20 mV to protons in the middle third of the pore, which has the effect of increasing the proton concentration in the core at the expense of potassium and simultaneously both increasing the proton selectivity and decreasing the ionic conductance. Thus the model gives a measure of the extent to which the diffusion of the ions must be restricted in a 10 Å pore in order to produce the selectivities and conductance that we measure.

If instead of the experimental diameter of 10 Å we use a diameter of 4 Å, akin to gramicidin, we can get a comparable fit to the data with D_{H^+} restored to its bulk value and $D_{K^+} = D_{Cl^-} = 8 \times 10^{-7}$ cm^2/s, which is only 1/24 times the bulk value. A PNP analysis of the gramicidin channel used a diffusion constant for KCl about 8 times lower than the bulk value (*29*). This value is consistent with MD simulations that found that the diffusion coefficients of K^+ and Cl^- were reduced in narrow channels by up to a factor of 10 (*30*). However, this same MD study found that the diffusion of these ions in larger pores, such at the Ach receptor, was comparable to bulk water and that the extent of degree of immobility in narrow pores was not sensitive to the secondary structure of the pore. Taken together, these results suggest that the flow of salt ions in the colicin A channel is more restricted than they are in the far smaller gramicidin channel and offer no hint as to what mechanism might be able to accomplish this.

Membrane Lipids Are Not the Cause of the High Proton Selectivity. There are several reasons to imagine that membrane lipids might be involved in the high proton selectivity we observe (*10*, *11*, *31*, *32*). First, and most simply, lipid molecules are suspected of contributing directly to the channel structure. The evidence for this is at best circumstantial, but the lack of any plausible all-protein model that can account for the known properties of the colicin channel is a forceful argument in its favor. Nevertheless, none of our findings here lends any support to this idea. If the channel were partly made of lipid, its properties might well be quite sensitive to the nature of the available lipid. While various effects on colicin behavior attributable to the lipids have been found, none of our lipid substitution experiments were able to eliminate the proton anomaly. The equivalence of PC/PI lipids to asolectin in our assay shows that none of the minor lipids in asolectin are important. Removing the PI, and thus the negative surface charge, and leaving a membrane of the synthetic, zwitterionic lipid diphytanoyl PC results in a small decrease in the H^+/K^+ selectivity, but the basic effect persists. The act of removing the surface charge should increase the surface pH as well as change the ion selectivity of the colicin, making the membrane more anion selective (*33*). Both of these effects would be expected to decrease the H^+ selectivity.

It has been suggested that negatively charged components of biological membranes can catalyze proton transport along the surface(*34*, *35*), although the practical importance of such an effect has been questioned (*36*). If the colicin forms a channel by distorting the bilayer structure in such a way as to bring its two surfaces into contact, then such a negative-charge-mediated surface proton conductance would become a transmembrane proton path and might explain our basic effect. The persistence of the effect in membranes made of pure PC or GMO says that it does not. This result does not rule out a structural role for lipid molecules in the channel or even prove that the channel does not create a continuity between the two membrane surfaces, but it does show that, even if lipids are involved, the possession of a negatively charged headgroup is unimportant in proton selectivity (although negative surface charge greatly enhances colicin activity). In addition, the persistence of the anomaly in GMO membranes rules out any requirement for phosphate (which, of course, can participate in protonation/deprotonation reactions, albeit at low pH) in the headgroup or even for a zwitterionic headgroup of any kind.

One can imagine a role for lipids in the proton conductance of colicin channels apart from their putative structural role in the channel. Perhaps the presence of open channels in the membrane modifies the adjacent membrane, or more likely the lateral protein/lipid interface, in such a way as to promote proton conductance through the bilayer. Protons are thought to permeate pure lipid membranes via transient water wires, or perhaps transient water clusters (*22*, *37*), and colicin might stabilize such structures. Even if the proton conductance that our data imply occurs outside of the channel proper, agents that modify bilayer proton permeability would be expected to modify the V_r that we measure. For example, sterols incorporated into a bilayer are known to modify its permeability to ions. The sterol ergosterol has been proposed to contribute to the inhibition of proton leakage across the membrane plant cells, which use a proton gradient for energy metabolism (*22*), but we found no effect of ergosterol on the colicin A V_r in pH gradients. Likewise, hydrocarbon sequestered in the bilayer may modify its proton conductance (*38*), yet we find no difference in V_r between

membranes containing squalene and those containing decane, even though the decane film contains more hydrocarbon (*39*). In summary, the lack of any significant effect on P_H/P_K in the various lipid manipulations studied argues against any direct role for lipid in the P_H/P_K anomaly.

Role of Histidine. Histdine residues, which are titratable at physiological pH, are prospective regulators of proton conductance. This was dramatically illustrated in the potassium channel, where it was shown that introducing a histidine residue into the voltage sensing domain, a part of the protein not normally involved in conduction, could convert that domain into a voltage-dependent proton channel or proton carrier, depending on the position of the histidine (*7*, *8*). Histidines have also been shown to regulate conductance in the M2 protein of influenza virus, perhaps the best understood proton channel. M2 is a tetramer of 95 aa in which one transmembrane helix from each subunit combines to form the narrow pore (*40*). His 37 is crucial for conductance in M2 (*41*−*43*), although its role appears to be structural rather than in direct regulation of the conductance pathway (*44*, *45*). Colicin A has three histidines in its channel-forming domain, whereas the other colicins tested have zero (colicin Ia) or one (colicins E1 and B). The two histidines in the upstream half of the domain were swapped out along with that subdomain, to no effect. Replacing the C-terminal histidine with cysteine had only a small effect, as did reacting the intact domain with DEPC. Taken together, these data strongly suggest that none of the histidines are required for the high proton selectivity.

Implications for the Structure of the Colicin Channel. Putting aside the question of mechanism, the anomalous proton selectivity can be used to address certain questions on the structure of the colicin channels. For colicins A, E1, and Ia, there is good evidence that channel formation does not require all of the 10-helical C-terminal domain (*12*, *17*, *46*). Colicin A and Ia have been shown to translocate a large part of themselves, roughly the second through fifth helix of the water-soluble structure, across the bilayer in association with gating (*12*, *20*). This translocated region presumably plays little role in the structure of the open channel, leaving only four potential transmembrane segments (H1, H6/7, H8, and H9) to form the pore. The hydrophobic hairpin (H8 and H9) has been shown to form two of them by cysteine scanning mutagenesis (*47*). We found that swapping H8 and H9 of colicin E1 into colicin A altered the proton selectivity of the colicin A host protein, making it more like E1, although it did not convert colicin A to colicin E1. In the case of colicin Ia, a role for helix 1 has been inferred from the observation that it too can be translocated in association with gating, under conditions where it is not prevented from doing so by an upstream anchor (a role normally fulfilled by upstream domains). The effect of such a translocation of helix 1 is to reduce the conductance of the channel by a factor of about 7, suggesting a role for it in the conductance pathway (*13*, *48*). The situation for colicin A is different. Deletion experiments have shown that the three upstream-most helices can be removed with little effect on conductance, while additional removal of the fourth and fifth helices leads to channels with altered properties (*17*). Helix 1 of colicin A does not undergo an Ia-type translocation in the channel formed by the 10-helix domain. Collectively, these observations suggest that the conduction pathway is formed from the five C-terminal helices along with one other transmembrane segment, which in the case of colicin Ia is H1 and in the case of colicin A is not. These observations lead to the question of what is the function of the first five helices? Does any part of their sequence form part of the normal conduction pathway? Our experiments here suggest that they do not. The experiments with the hybrid colicins and the truncated colicin A construct are all consistent with assigning the anomalous proton conductance to the C-terminal five helices. Gross alterations in helices 1−5, as represented by these constructs, do not alter P_H/P_K, whose anomalous nature in this system implicates it as a sensitive indicator of the structure of the conduction pathway. This inference is bolstered by the trans trypsin experiments. Trans trypsin is presumably acting, at least in the case of Ia, by cutting the protein in the translocated region (helices 2−5) in the open sate, effectively creating a truncated channel lacking helix 1. This increases P_H/P_K by a factor of 2, which is less than would be expected based simply on the 7-fold drop in KCl conductance engendered by the enzyme. It does not convert a colicin Ia P_H/P_K to a colicin A-like P_H/P_K, even though it does reduce the single channel conductance to a value close to that of colicin A. Taken together, these results support the concept of a three-transmembrane core channel structure, with the fourth, upstream transmembrane segment acting as a peripheral influence on channel properties.

Conclusions. In this paper we have attempted to relate the anomalous proton selectivity of colicin A reported previously (*1*) to certain known and speculative features of the channel. We sought evidence for a separate proton pathway but were unable to find any. For lack of sufficient protein to model the channel with a lumen of the appropriate diameter, lipid molecules have been proposed as structural elements. If lipids are so employed, changing the available lipids would be expected to alter channel properties, presumably including the proton selectivity. Our finding that the proton selectivity is insensitive to gross changes in the membrane lipids argues against such models. A corollary to this inference is that lipids and proteins cannot form separate pathways for protons and other ions. Nor can protons be using the lipid/protein interface as a high capacity conduit. The results with the hybrid colicins and with trypsin firmly locate the selectivity determinants to the C-terminal half of the channel-forming domain.

Since the conduction pathway accommodates large ions and yet discriminates in favor of protons, it must harbor a mechanism to reduce the mobility of ions in the pore. The particular permeation model used predicted a 1000-fold reduction, but in any case it is substantial. Indeed, it may be more accurate to describe the ionic selectivity as anomalously low, rather than to describe the proton selectivity as anomalously high. Various mathematical schemes to account for this are feasible, such as binding models in which a proton binds to a site in the channel, blocking the lumen to ion conductance before exiting, but it is unclear how this can be accomplished in a wide lumen. An alternative mechanism, perhaps no less fanciful, posits that the lumen possesses a narrow, highly selective bottleneck that occasionally flickers open, on a time scale yet to be observed, so as to allow large ions to pass.

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