Identification of a Translocated Gating Charge in a Voltage-dependent Channel

Colicin E1 Channels in Planar Phospholipid Bilayer Membranes

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ABSTRACT The availability of primary sequences for ion-conducting channels permits the development of testable models for mechanisms of voltage gating. Previous work on planar phospholipid bilayers and lipid vesicles indicates that voltage gating of colicin E1 channels involves translocation of peptide segments of the molecule into and across the membrane. Here we identify histidine residue 440 as a gating charge associated with this translocation. Using site-directed mutagenesis to convert the positively charged His440 to a neutral cysteine, we find that the voltage dependence for turn-off of channels formed by this mutant at position 440 is less steep than that for wild-type channels; the magnitude of the change in voltage dependence is consistent with residue 440 moving from the *trans* to the *cis* side of the membrane in association with channel closure. The effect of *trans* pH changes on the ion selectivity of channels formed by the carboxymethylated derivative of the cysteine 440 mutant independently establishes that in the open channel state, residue 440 lies on the *trans* side of the membrane. On the basis of these results, we propose that the voltage-gated opening of colicin E1 channels is accompanied by the insertion into the bilayer of a helical hairpin loop extending from residue 420 to residue 459, and that voltage-gated closing is associated with the extrusion of this loop from the interior of the bilayer back to the *cis* side.

INTRODUCTION

Various mechanisms have been proposed to account for the gating of ion-permeable channels by transmembrane voltage (see, for example, Hille, 1984). It has long been recognized that voltage control of a process formally implies that charges must move through the electric field, and that the steepness of the voltage dependence is a measure of the total amount of work done on the charges responsible for the

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voltage-dependent effect. The voltage dependence displayed by a few small, channel-forming molecules, such as monazomycin and melittin, is thought to result from voltage-driven translocation of charges across the entire membrane (Heyer et al., 1976; Kempf et al., 1982), whereas the voltage dependence of integral membrane channels, such as the neuronal sodium channel, is thought to involve many individual charges (or dipoles) moving a small distance within the membrane (e.g., Armstrong, 1981). In this paper we identify one of the gating charges of colicin E1, a voltage-dependent channel formed by a large protein, and suggest that, in this instance, gating is associated with a substantial transmembrane movement of this charge.

The colicins are a group of plasmid-encoded bacterial toxins expressed by different strains of *E. coli*. Those of the E1 subgroup, which includes colicins A, N, B, Ia, Ib, and E1 itself, form ion-permeable channels both in the inner membrane of the target bacterium and in lipid bilayer membranes. Each of these channel-forming toxins, all of which have been sequenced, consists of a polypeptide chain of between 500 and 600 amino acids, with a single hydrophobic segment (residues 474–509 in colicin E1) near the carboxyl terminus. For colicins E1 and A, it has been shown explicitly that only the carboxyl terminal region, which includes the hydrophobic segment, is required for channel formation. (For a general review of the channel-forming colicins, see Pattus et al., 1990.)

The addition of colicin E1 in nanomolar concentrations to the bathing solution on one side of a planar lipid bilayer (the *cis* side) induces a large, voltage-dependent conductance attributable to the gating of colicin channels. *Cis* positive voltages[1] tend to turn channels on, whereas *cis* negative voltages tend to turn them off (Cleveland et al., 1983). The colicin E1-induced bilayer conductance is also highly pH dependent, both in ion selectivity and in gating. At symmetric pH 4.1 the induced conductance is anion selective, but as the pH is raised symmetrically to 5.5 or higher it becomes cation selective (Raymond et al., 1985). In addition, increasing the pH of the *cis* bathing solution dramatically decreases the channel turn-on rate, whereas raising the *trans* pH dramatically decreases the turn-off rate (Raymond et al., 1986).

Studies both on planar lipid bilayers and on lipid vesicles suggest that voltage gating of colicin E1 channels involves translocation of peptide segments of the molecule across the membrane (Raymond et al., 1986; Slatin et al., 1986; Xu et al., 1988). Using site-directed mutagenesis to identify groups that are translocated with voltage gating, we show in this paper that changing a positively charged residue, histidine 440, to a neutral cysteine markedly reduces the voltage dependence of the turn-off rate kinetics of colicin E1 channels, and that the reduction is consistent with this group being translocated across the bilayer during voltage gating. Moreover, measurements of ion selectivity as a function of *trans* pH independently place residue 440 on the *trans* side of the bilayer in the open channel. These findings provide direct evidence for, and identification of, a "gating charge" which moves through the transmembrane field as part of the gating mechanism of a voltage-dependent channel.

[1] All voltages refer to those of the *cis* compartment with respect to that of the *trans*.

MATERIALS AND METHODS

Construction of Mutants and Their Chemical Modification

The mutant colicins, one with a cysteine residue substituted for the histidine at position 440, and one with cysteine substituted for threonine at position 490, were constructed by oligonucleotide mutagenesis as described previously (Jakes et al., 1990). Mutagenesis was performed on plasmid pKSJ22, which is pKSJ11 (Jakes et al., 1990), in which the colicin E1 gene was mutated so that the single cysteine at position 505 in wild-type colicin E1 was replaced by alanine.

The mutagenic oligonucleotides, synthesized on a 380A DNA synthesizer (Applied Biosystems, Inc., Foster City, CA), were as follows (with the mutated bases underlined):

for His440 → Cys: 5′ CCAAAAGAAACACACCCCG 3′

for Thr490 → Cys: 5′ CCTAATGTACATCCAGC 3′

The resulting colicins thus each had a single cysteine residue at the position specified, and will be designated C440/A505 and C490/A505. The mutant colicins were purified exactly as described in Jakes et al. (1990) for whole colicin molecules. The yield of C440/A505 was 25 mg/liter, with an activity of 1.5×10^5 U/mg; the yield of C490/A505 was 13 mg/liter, with an activity of 1.8×10^4 U/mg.

Carboxymethylation and carboxamidomethylation reactions of C440/A505 with iodoacetic acid and iodoacetamide were carried out as described previously (Jakes et al., 1990). The reactions of C490/A505 with iodoacetic acid and iodoacetamide were done in the presence of 6 M urea; in the absence of urea, there was no appreciable reaction of C490/A505 with [^{14}C]iodoacetamide.

Experiments in Planar Lipid Bilayers

Planar phospholipid bilayers were formed at room temperature by the union of two lipid monolayers (Montal, 1974) of asolectin (lecithin type IIS from Sigma Chemical Co., St. Louis, MO) from which neutral lipids were removed by the method of Kagawa and Racker (1971). Monolayers were spread from 1% solutions of lipid in hexane, and after evaporation of the hexane, membranes were formed across an 80–120-μm hole, pretreated with squalene (Reyes and Latorre, 1979), in a Teflon partition separating two compartments (~1 ml) containing buffered salt solutions. With the exception of the ion selectivity experiments, these solutions contained 1 M KCl, 5 mM $CaCl_2$, 1 mM EDTA, and 1 mM potassium glycerate (pH 3.5). Colicin-induced ion selectivity was determined by measuring reversal potentials in the presence of a 1 M to 0.1 M transmembrane KCl gradient; both solutions also contained 5 mM $CaCl_2$, 0.5–1.0 mM EDTA, and buffers as indicated in Table I. The membrane was formed in the presence of the gradient, and wild-type or mutant colicin was then added to the high salt side. Reversal potentials were measured by determining the voltage at which the rate of current change (dI/dt) reversed sign while channels were opening. Gradients were routinely checked at the end of the experiment by adding enough valinomycin to overwhelm the colicin-induced conductance, thereby making the membrane ideally K^+ selective. Electrical measurements were made under voltage clamp conditions as previously described (Jakes et al., 1990). For measuring turn-on and turn-off rates, currents were filtered at 2 kHz, digitized at 94 kHz with a 14 bit A/D converter (model VR-10 digital data recorder; Instrutech Corp., Mineola, NY), and recorded and stored on VHS tape (Panasonic PV-2700 VCR) for subsequent playback and analysis.

Voltage dependence of turn-off rates. After sufficient numbers of wild-type or mutant colicin E1 channels were incorporated, the membrane was pulsed to +30 mV to turn channels on and then pulsed to a given voltage, ranging from −80 to +10 mV, to observe their rate of turn-off as reflected in the rate of current decay (see Fig. 1). Because the absolute rates of turn-off tend to slow down after many minutes (possibly because of channel aggregation), an effort was made to collect all of the data within the first 2–3 min of the experiment. Using a digitizing oscilloscope (Explorer oscilloscope; Nicolet Instrument Corporation, Madison, WI) to resolve current decay with time, we subtracted baseline (leak) current and plotted the resulting colicin-induced current semilogarithmically as a function of time (see Fig. 2 *A*). If the turn-off process is characterized by a single exponential, such a plot, at a given voltage, is a straight line whose slope reflects the time constant (τ_{off}) for turn-off at that voltage. A plot of τ_{off} vs. V for voltages at which current decays virtually to zero yields the voltage dependence of the turn-off rate constant (k_{-1}), since in this regime $\tau_{off} \approx 1/k_{-1}$.

For colicin E1 channels, turn-off kinetics are not so simple and are dependent on at least two separate voltage-dependent time constants; moreover, the relative contributions of these time constants to the kinetics are also voltage dependent. At the most negative voltages considered ($V \leq -40$ mV), the fastest time constant predominates; at less negative voltages the slower time constant becomes more significant until, at the least negative voltages considered, it predominates. One can therefore determine the voltage dependence of the fast or slow component by considering the appropriate voltage region, thereby obviating the need for complicated curve fitting procedures to separate the two time constants. In this paper we concentrate on the faster time constant, which predominates at $V \leq -40$ mV; the voltage dependence of τ_{off} was obtained from semilogarithmic plots of τ_{off} vs V.[2]

Voltage dependence of turn-on rates. Because colicin E1-induced conductances in planar lipid bilayers rarely reach a steady state, turn-on time constants and their voltage dependencies cannot be directly measured. However, if one assumes a two-state process for gating (which, for colicin E1, is not totally accurate [see Discussion]),

$$C \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} O \tag{1}$$

and if all channels are initially in the closed state, i.e., $[o]_{t=0} \approx 0$, then initially

$$d[o]/dt = k_1[c]$$

or

$$dg/dt = (g_{\text{single channel}})d[o]/dt = (g_{\text{single channel}})k_1[c]$$

where g is the colicin-induced conductance. Therefore, if the total number of channels (open plus closed) in the membrane remains constant, and all the channels are initially closed, dg/dt will be linear and proportional to k_1 at short times after turn-on test pulses are applied. In experiments to measure the voltage dependence of k_1, the turn-on rate constant, sufficient wild-type or mutant colicin E1 was added to the *cis* compartment to insure large conductances, so that subsequent linear increases in conductance for short times (in response to positive voltage pulses) represented only a small fraction of the total possible membrane conductance. Voltage was alternated between −60 mV pulses of ~5 s, to turn all channels off, and a series of

[2] Although in this voltage range the data are fit by a single exponential with time constant τ_{off}, it is possible that this exponential is contaminated by an unresolvable slow component, as seen for $V > -40$ mV. Extrapolating this slow component into the region $V \leq -40$ mV and using it to correct τ_{off}, we found no significant change in τ_{off} voltage dependence. For this reason, and since in any case this correction is model dependent, we have refrained from such data massage.

turn-on pulses, from +20 to +80 mV. Initial turn-on rates were determined from the slope $[(dI/dt)_{t=0}]$ of the I vs. t line at short times (see Fig. 3). (At the end of a series, the earliest pulses were repeated to correct for any upward drift in initial rates resulting from continuing channel incorporation. If this correction was >25%, the data were not used and the pulse routine was repeated; in most cases, the correction was <10%.) $(dI/dt)_{t=0}$ was converted to $(dg/dt)_{t=0}$ and plotted semilogarithmically as a function of voltage. For voltages lying between +15 and +40 mV, the $\log(dg/dt)_{t=0}$ vs. V curve was linear, and its slope was fitted using a nonlinear least-squares fitting program appropriate for an exponential function (micro TSP; Quantitative Micro Software, Irvine, CA), thus yielding the voltage dependence of k_1.

RESULTS

Voltage Dependence of Turn-off Rates

The current responses of wild-type, or mutant, colicin E1-modified membranes to a series of voltage pulses that close the channels were obtained and plotted semiloga-

TABLE I

Reversal Potentials (in Millivolts) of Wild-Type and Mutant Colicin E1 Channels in a 10:1 KCl Gradient across Asolectin Membranes

Buffer conditions		Colicins		
cis	*trans*	C440/A505	C440/A505 carboxymethylated	Wild-type
1 mM glycerate pH 4.1	1 mM glycerate pH 4.1	27.3 ± 2 (6)	23 ± 1.5 (5)	32 ± 2.5 (4)
1 mM glycerate pH 4.1	1 mM glycerate pH 3.4		35 ± 0.8 (3)	35 ± 0.5 (2)
10 mM glycerate pH 4.1	1 mM EDTA pH 5.0		7.5 ± 4 (4)	25 ± 1.7 (4)
100 mM glycerate pH 4.1	1 mM EDTA pH 5.0		11 ± 4.3 (3)	28 ± 1.3 (3)
1 mM EDTA pH 5.0	50 mM glycerate pH 3.4		25 ± 2.8 (2)	24 ± 2.8 (2)

The *cis* and *trans* solutions contained 1 M KCl and 0.1 M KCl, respectively. Reversal potentials, all of which are positive, are that of the *cis* compartment with respect to that of the *trans*. The number of experiments is given in parentheses.

rithmically as described in Materials and Methods (e.g., Figs. 1 and 2 *A*). Closing rates are voltage dependent (Fig. 2, *A* and *B*), and between −80 and −40 mV, the region of interest, the fraction of channels remaining open decreases exponentially with time with a voltage-dependent time constant, τ_{off}. (At less negative voltages a second slower process appears, which actually dominates the overall turn-off rate at $V \geq -10$ mV.) Semilogarithmic plots of τ_{off} vs. V yield straight lines. For a given form of E1 (wild-type, mutant, or carboxymethylated mutant) the absolute value of τ_{off} at a given voltage can vary from membrane to membrane by as much as a factor of 5, but the slope of the $\log(\tau_{off})$ vs. V line is quite constant: for wild-type, e-fold per 20 ± 2.3 mV (n = 4; range, 17.5–23 mV); for C440/A505 mutant, e-fold per 42 ± 8.1 mV (n = 5; range, 34–50 mV). The slope of the log (τ_{off}) vs. V line for the carboxymeth-

ylated C440/A505 mutant, *e*-fold per 35 ± 1.4 mV (n = 2; range, 34–36 mV), is not significantly different from that of unreacted C440/A505. The difference in τ_{off} voltage dependence between wild-type and C440/A505 channels is illustrated in Fig. 2 *B*.

The voltage dependencies of two other mutants were determined. For C473/A505, a mutant in which a wild-type aspartate has been replaced by a cysteine and in which an altered pH dependence of selectivity is seen (Jakes et al., 1990), the voltage dependence is *e*-fold per 25 mV (n = 1); for C490/A505, a mutant in which a wild-type threonine is changed to cysteine, the voltage dependence is *e*-fold per 20 mV (n = 1). Thus, channels formed by these other mutants exhibit essentially wild-type voltage dependence for τ_{off}, suggesting that it is the specific removal of the positive charge at residue 440, and not some nonspecific property of colicin mutants, which is responsible for the shallower voltage dependence of τ_{off} for C440/A505 and carboxymethylated C440/A505 channels compared with that of wild-type channels.

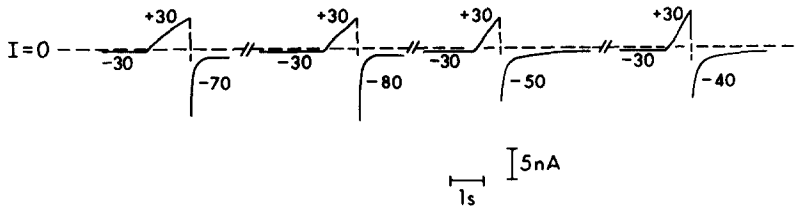
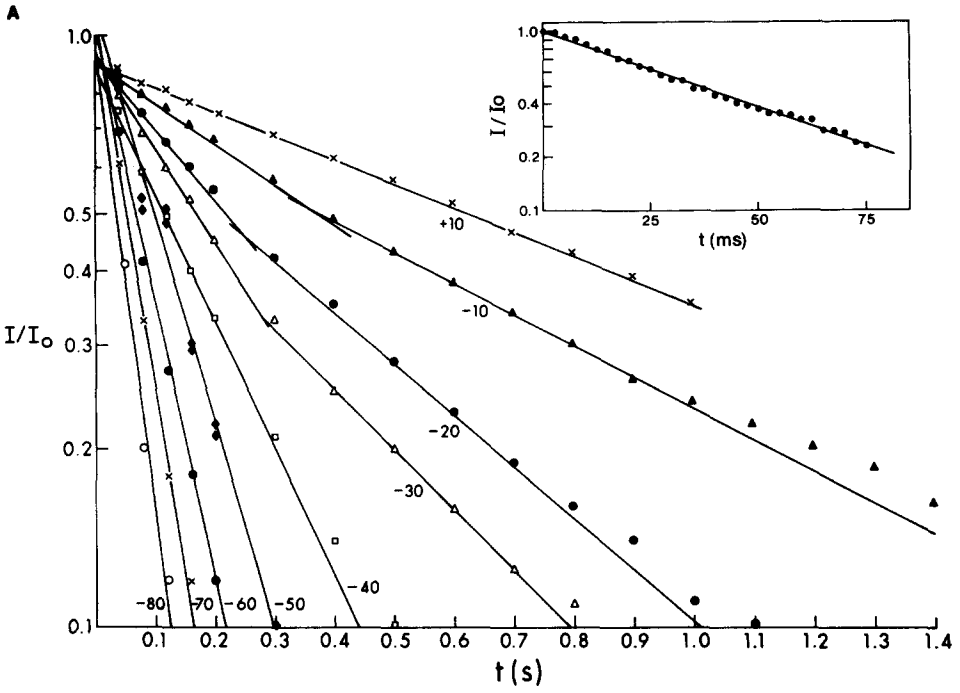


FIGURE 1. Turn-off of colicin E1-induced conductance at negative voltages. Colicin E1 mutant C440/A505 was added to a concentration of ~1 μg/ml to the *cis* compartment 30 s before the beginning of the record. The membrane was then alternately pulsed between +30 mV to turn channels on, and various negative voltages to turn them off; the breaks in the record are of ~30 s duration. Note that for turn-off, which is a fast process, current as a function of time cannot be directly determined from a chart-recorded tracing such as this one. For this reason the current trace was recorded and digitized as described in Materials and Methods. Membranes were formed from asolectin in 1 M KCl, 5 mM $CaCl_2$, 0.5 mM EDTA, and 1 mM potassium glycerate, pH 3.5.

This shallower voltage dependence is consistent with residue 440 moving in a *trans* to *cis* direction during turn-off gating (see Discussion).

Voltage Dependence of Turn-on Rates

Fig. 3 shows responses of a colicin E1-treated membrane to a series of turn-on pulses, separated by −60-mV turn-off pulses of ~5 s (which at pH 3.5 is long enough to turn off essentially all of the colicin-induced conductance). To a first approximation (but see Discussion) current initially increases linearly with time in response to a turn-on pulse. From data such as these, semilogarithmic plots of the initial rate of conductance increase $[(dg/dt)_{t=0}]$ versus voltage were constructed (e.g., Fig. 4). Within experimental error, there is no difference at pH 3.5 between the voltage dependence of the turn-on rate of C440/A505, its carboxymethylated derivative, wild-type, and C473/A505 channels (*e*-fold changes in $[dg/dt]_{t=0}$ for 11.3 ± 2.1 mV [n = 6], 11.0 ± 0.8 mV [n = 5], 10.7 ± 1.8 mV [n = 5], and 10.1 mV [n = 1], respectively).



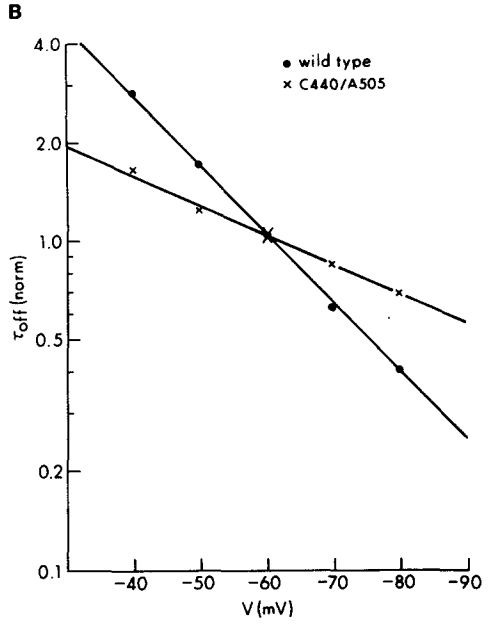


FIGURE 2. (*A*) Measurements of turn-off kinetics at several different voltages. The data are from the same experiment as in Fig. 1. Currents were digitized, background current was subtracted, and the normalized current I/I_o (where I_o is the colicin-induced current at $t = 0$) was plotted as described in Materials and Methods. Note that at voltages ≤ -50 mV, the log (I/I_o) curves are linear, while at less negative voltages a slower component appears. At the least negative voltages (−10 mV and +10 mV) the response is dominated by the slower component. At voltages for which there is one component, the slope of the line reflects the turn-off time constant, τ_{off}. (*Inset*) The first 75 ms of the −80-mV record plotted on an expanded time scale. (*B*) Comparison of voltage dependence of τ_{off} for wild-type and C440/A505 channels. For each separate experiment ($n = 4$ for wild-type and $n = 6$ for C440/A505), the values of τ_{off} at each voltage were normalized to the τ_{off} at −60 mV, and then plotted as shown. It can be seen that the voltage dependence of τ_{off} for the mutant channel (*e*-fold change per 47.5 mV) is substantially less steep than that for wild-type (*e*-fold change per 20 mV). Error bars have been omitted for clarity. For the wild-type experiments the standard deviations at each voltage were <21% of the average values, while for the C440/A505 experiments deviations were at most 15%.

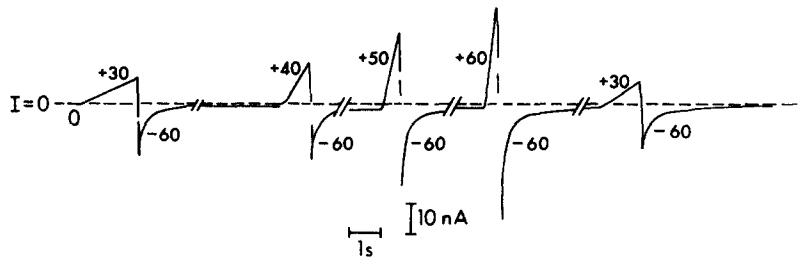


FIGURE 3. Turn-on of colicin E1-induced conductance at positive voltages. Wild-type colicin E1 was added to a concentration of 1 μg/ml to the *cis* compartment and allowed to equilibrate with the membrane for ~3 min. The membrane was then alternately pulsed between various positive voltages, to obtain a turn-on rate, and −60 mV to turn off all conductance. Successive breaks in the record indicated intervals of 5, 3, 5, and 5 s, respectively. Conditions are as in Fig. 1.

Ion Selectivity

To further localize the position of residue 440 in the open colicin E1 channel, the pH dependence of the selectivity of C440/A505, carboxymethylated C440/A505, and wild-type channels was examined. Table I lists the reversal potentials in a 10-fold KCl gradient for mutant and wild-type channels under various pH and buffer conditions. At symmetric pH 4.1 there is a small difference between the anion selective reversal potential of C440/A505 channels (27 ± 2 mV [n = 6]) and that of wild-type channels (32 ± 2.5 mV [n = 4]). When a carboxyl group is introduced at position 440 by carboxymethylation of C440/A505, the reversal potential drops further to 23 ± 1.5

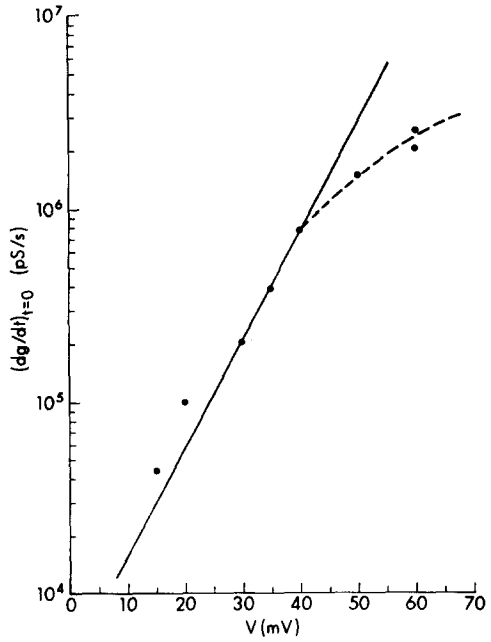


FIGURE 4. The voltage dependence of the turn-on rate of colicin E1 channels. This figure shows a plot of the initial rate of conductance increase ($(dg/dt)_{t=0}$) as a function of stimulating voltage. $(dg/dt)_{t=0}$ at each voltage was calculated as described in Materials and Methods from records such as those shown in Fig. 3. Note that for $V \geq 50$ mV, the slope of the $(dg/dt)_{t=0}$ vs. V curve begins to flatten out. Points between 15 and 40 mV were fit with a nonlinear least-squares algorithm as described in Materials and Methods. The slope of the best-fit line then yields the voltage dependence of the turn-on rate constant, k_1. In this case, for an experiment with wild-type colicin E1, k_1 changes e-fold per 8 mV. Conditions are as in Fig. 1.

mV ($n = 5$). Since this carboxyl group is titratable, the difference in reversal potentials for carboxymethylated C440/A505 and wild-type channels should be a function of the pH in the local environment of residue 440. From the turn-off rate experiments we have previously inferred that this residue is near the *trans* compartment, and this is indeed confirmed in experiments measuring reversal potentials. When the *trans* pH is lowered to 3.4, the reversal potentials for wild-type and carboxymethylated C440/A505 channels become virtually identical, 35 ± 0.5 mV ($n = 2$) and 35 ± 0.8 mV ($n = 3$), respectively, whereas when the pH of the *trans* compartment is raised to 5.0, reversal potentials now differ by ~17 mV (Table I).

If residue 440 is actually exposed to the *trans* bath, then at a given *cis* pH, the difference in reversal potentials between wild-type and carboxymethylated C440/A505 mutant channels should be dependent only on the *trans* pH, and not on the relative buffering capacities of the *cis* and *trans* solutions. On the other hand, if this residue is near the *trans* side but within the lumen of the channel, and hence accessible to channel-permeant *cis* buffer, the difference in reversal potentials should be reduced as the *cis* buffer capacity is increased to levels much higher than that on the *trans* side. The titratable carboxyl group introduced chemically at residue 440 by carboxymethylation of C440/A505 does not appear to "feel" the *cis* pH, even when the *cis* buffer capacity is 20 or even 100 times greater than that of the *trans* side (Table I). In fact, under *cis* pH 4.1/*trans* pH 5.0 conditions, with a 20- or 100-fold excess of permeant buffer on the *cis* side, the difference between the ion selectivity of the carboxymethylated C440/A505 channels and that of wild-type channels is greater than it is at symmetric pH 4.1. (At symmetric pH 4.1, reversal potentials of wild-type and carboxymethylated C440/A505 channels differ by 9 mV; at *cis* pH 4.1/*trans* pH 5.0, and *cis* buffering capacity 100 times that of *trans*, the difference is ~17 mV. Specifically, in going from symmetric pH 4.1 to *cis* pH 4.1/*trans* pH 5.0, with *cis* buffering capacity 100 times that of *trans*, the anion selective reversal potential of wild-type channels declined by only 4 mV, whereas that of the carboxymethylated C440/A505 mutant channels declined by 12 mV.) Taken as a whole, these selectivity measurements demonstrate that carboxymethylated C440/A505 channels possess a titratable group on the *trans* side of the membrane not present in wild-type channels. Moreover, under *cis* pH 5.0/*trans* pH 3.4 conditions, there is no discernable difference in ion selectivity between carboxymethylated C440/A505 channels and wild-type channels (Table I). Thus, this titratable group is not also present on the *cis* side of the membrane, as it could conceivably be if, for example, the channel is a dimer with residue 440 facing the *trans* side in one monomer and the *cis* side in the other.

DISCUSSION

Previous studies on planar lipid bilayers and lipid vesicles have indicated that voltage gating of colicin E1 channels involves translocation of parts of the protein across the membrane, exposing different domains to the *cis* and *trans* solutions in the open and closed states (Raymond et al., 1986; Slatin et al., 1986; Xu et al., 1988). In this paper we compared the voltage dependence of gating kinetics between wild-type channels and those formed by a mutant in which the histidine at residue 440 was replaced by cysteine. We found that residue 440 is translocated through the membrane from the

trans side toward the *cis* side when the channels are turned off by voltage; by inference, it is translocated from the *cis* to the *trans* side when the channels are turned on by voltage. From a comparison of ion selectivities between wild-type channels and those formed by the carboxymethylated derivative of the mutant protein, which has a titratable carboxyl group at residue 440, we further demonstrated that in the open channel this residue lies fully exposed to the *trans* solution, rather than lying within the channel lumen or buried within the lipid bilayer. Before discussing the implications of these findings for channel gating and structure, let us review the evidence that leads us to the above assertions concerning the location and sojourns of residue 440.

Inferences from Ion Selectivity

In a 10:1 KCl gradient at symmetric pH 4.1, channels formed by the carboxymethylated C440/A505 mutant have a reversal potential 9 mV less anion selective than those formed by wild-type E1, presumably because the titratable carboxyl group at residue 440 retains some negative charge at this pH.[3] If this residue is exposed to the *trans* solution when the channel is open, the difference in ion selectivity between wild-type and carboxymethylated C440/A505 channels (at a fixed *cis* pH) ought to be controlled exclusively by the *trans* pH, independent of the concentration of channel-permeant buffer in the *cis* solution (since the residue can sense only the *trans* pH). (In contrast, if the 440 residue resides within the channel, even though close to the *trans* side, it would sense the *cis* pH if the permeant buffer concentration in the *cis* solution is high compared with that of the *trans* solution.) We have found (see Table I) that even with a 100-fold excess of permeant buffer in the *cis* solution, the reversal potential of carboxymethylated C440/A505 channels changes (becomes less anion selective) by 24 mV when the *trans* pH is increased from 3.4 to 5.0, whereas the reversal potential of wild-type channels changes by only 7 mV. This result strongly argues that the carboxyl group introduced at residue 440 is exposed to the *trans* solution and does not sense the luminal pH.

Inferences from Voltage Dependence of Gating

At pH 3.5, the pH at which all gating experiments were conducted, it is reasonable to assume that His440 is fully positively charged (the pK of free histidine is 6.0). The replacement of this histidine by a cysteine ought therefore to result in the C440/A505 mutant having one less positive charge than wild-type E1. (At pH 3.5 it is also reasonable to assume that the net charge on a carboxyl group is very small, and in fact the selectivity measurements suggest that the carboxyl group of carboxymethylated Cys440 is uncharged at this pH.) Thus, if during voltage-dependent gating the 440 residue moves through the electric field imposed across the bilayer, one expects to see a reduced voltage dependence of the rate constants when the positively charged histidine at this position is removed.

[3] The positive charge of His440 in wild-type colicin E1 apparently does not significantly influence the ion selectivity of the channel, as is evidenced by the marginal difference in reversal potentials between wild-type and C440/A505 channels (Table I).

Formally, for a two-state open to closed transition:

$$C \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} O \tag{1}$$

one can write for the rate constants

$$\begin{aligned} k_1 &= A \exp(-\Delta G_1^{\ddagger}/kT) \\ k_{-1} &= A \exp(-\Delta G_{-1}^{\ddagger}/kT) \end{aligned} \tag{2}$$

where the $\Delta G^{\ddagger}$'s are the activation free energy differences for the closed to open and open to closed transitions, respectively, A is a constant, k is the Boltzmann constant, and T is temperature in degrees Kelvin (see, for example, Ehrenstein et al., 1974). If gating involves the movement of n charges (of unit charge q) in the electric field applied across the membrane, one can further expand the $\Delta G^{\ddagger}$'s into chemical and electrical parts:

$$\Delta G^{\ddagger} = (\Delta G^{\ddagger})_{\text{chem}} + nq\delta V$$

so that,

$$\begin{aligned} k_1 &= A \exp\{-[nq\delta_{\text{on}}V + (\Delta G_1^{\ddagger})_{\text{chem}}]/kT\} \\ k_{-1} &= A \exp\{-[nq\delta_{\text{off}}V + (\Delta G_{-1}^{\ddagger})_{\text{chem}}]/kT\} \end{aligned}$$

where $nq\delta_{\text{on}}$ and $nq\delta_{\text{off}}$ are the effective charges moved in opening and closing the channels, respectively, and $(\Delta G^{\ddagger})_{\text{chem}}$ is the chemical (i.e., non–voltage dependent) contribution to the activation energy. (If a unit charge q moves completely across the membrane, then $n = 1$ and $q\delta_{\text{on}} + q\delta_{\text{off}} = q$; that is, δ_{on} is then the electrical distance from the *cis* side to the energy barrier for q, δ_{off} is the electrical distance from the *trans* side to the energy barrier for q, and $\delta_{\text{on}} + \delta_{\text{off}} = 1$.) Since we are concerned only with the voltage dependence of the rate constants, and $\exp(-\Delta G_{\text{chem}}^{\ddagger}/kT)$ is voltage independent, then

$$\begin{aligned} k_1 &= A' \exp(-nq\delta_{\text{on}}V/kT) \\ k_{-1} &= A'' \exp(-nq\delta_{\text{off}}V/kT) \end{aligned} \tag{3}$$

where $A' = A \exp[-(\Delta G_1^{\ddagger})_{\text{chem}}]$ and $A'' = A \exp[-(\Delta G_{-1}^{\ddagger})_{\text{chem}}]$.

For the wild-type channel the voltage dependence of k_{-1}, the turn-off rate constant, is an e-fold change per 20 mV (Fig. 2 *B*), making (since $kT/q = 25.6$ mV) $nq\delta_{\text{off}}$ equal to 1.28 effective charges; for the C440/A505 mutant channel the voltage dependence of k_{-1} is an e-fold change per 41 mV (Fig. 2 *B*) or $nq\delta_{\text{off}} = 0.62$ effective charges, whereas for its carboxymethylated derivative the corresponding numbers are e-fold change per 35 mV or $nq\delta_{\text{off}} = 0.73$ effective charges. (The C440/A505 and carboxymethylated C440/A505 results are the same within experimental error.) Thus, ~ 0.6 more effective charges move in closing the wild-type channel than in closing the mutant or carboxymethylated mutant channel. Assuming that the His440 residue is translocated across the membrane in channel gating (i.e., it is on the *trans* side in the open state and on the *cis* side in the closed), this suggests that the barrier to its

translocation is located an electrical distance of 0.6 from the *trans* side. In other words, assuming a constant electric field, the energy barrier for the translocation of His440 lies roughly in the middle of the bilayer, just where one anticipates it to lie for a charge moving through the hydrophobic interior of the bilayer.

If His440 moves from the *trans* to the *cis* side during channel closing, it must move from the *cis* to the *trans* side during channel opening. Consequently, removing the charge at residue 440, as was done in constructing C440/A505, ought to make the voltage dependence of turn-on less steep than in wild type. Although, within experimental error, we find no difference between the voltage dependence of turn-on rates for wild-type and C440/A505 mutant channels, this is not inconsistent with translocation of residue 440 during gating. The voltage dependence of k_1, the turn-on rate constant, is an e-fold change per 10 mV for wild-type channels. From Eq. 3, this makes $nq\delta_{on}$ equal to 2.56 effective charges. If the barrier to translocation of the histidine is located an electrical distance of 0.4 from the *cis* side, $nq\delta_{on}$ for

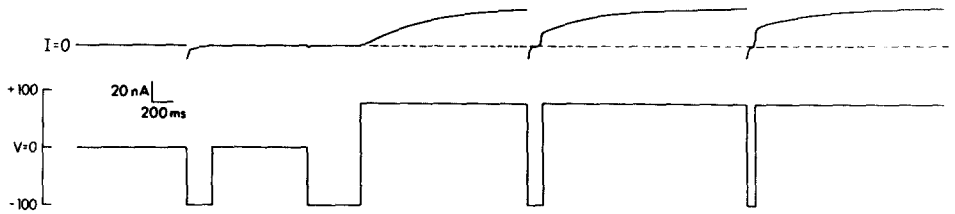


FIGURE 5. Response of a colicin E1-treated membrane to negative voltage pulses of short duration. The lower trace is the applied voltage and the upper trace is the current response. The bilayer was made of diphytanoyl phosphatidylcholine and plant phosphatidylinositol (4:1 by weight). *Cis* solution was 1 M NaCl, 5 mM $CaCl_2$, and 20 mM dimethylglutaric acid (DMG), pH 3.5; *trans* solution was 1 M NaCl, 5 mM $CaCl_2$, and 20 mM DMG, pH 2.7. 20 ng of the thermolysin fragment of colicin E1 was added to the *cis* compartment 7 min before this section of the record. The chart recorder can barely resolve the rapid turn-off of the conductance in response to the −100-mV pulses. Subsequent turn-on at +100 mV has a fast component and a slow component.

C440/A505 channels would become 2.16 effective charges, and the voltage dependence of k_1 would then be an e-fold change per 12 mV, a difference of only 2 mV from the value of 10 mV for wild-type channels. The scatter in our data is too great to detect so small a change.

For the purposes of our analysis, we have assumed that the voltage-dependent gating of colicin E1 channels is described by a two-state system. This is, however, an oversimplification. The following observations argue that there are *at least* two closed channel states: (*1*) Turn-off kinetics are fit by a single exponential only at large negative voltages; at less negative voltages, at least two exponentials are required to fit the data (Fig. 2 *A*). (*2*) Turn-on kinetics are not consistent with a two-state system, as clearly illustrated in Fig. 5. (In this experiment, voltage protocols and pH conditions were chosen to emphasize the existence of at least two closed states, but we have no reason to believe that the basic gating mechanism is qualitatively different here than under our usual conditions.) When channels are turned off by a brief, large

negative voltage, the turn-on kinetics appear biphasic, suggesting that some of the previously turned off channels were in a shallow off state, while others were in a deeper state. Note that the shorter the off pulse, the larger the fraction of channels turning on from the shallow off state. The complications of the turn-on kinetics are further illustrated in Fig. 5, where the turn-on response is seen not to be exponential, but rather sigmoidal in form. The existence of multiple closed states makes it difficult to give an unambiguous *quantitative* interpretation to the shallower voltage dependence of the turn-off kinetics for C440/A505 channels, but it does not detract from our primary conclusion that residue 440 is translocated across the membrane during voltage gating.

Implications for Channel Structure and Gating

Two conclusions can be drawn from the work described here: (*1*) in the open channel state, residue 440 is located on the *trans* side of the bilayer; and (*2*) during voltage-dependent gating, residue 440 moves a significant distance, probably all the way, through the bilayer. The first result places significant constraints on modeling of the open channel structure, while the second has implications for the mechanism of voltage gating.

1. Open channel structure. In addition to the assignment of residue 440 to the *trans* side of the bilayer, several other facts must be incorporated into any model of the open colicin E1 channel. First, it has recently been shown, by infrared spectroscopy (Rath et al., 1990), that the membrane-bound form of the COOH-terminal fragment of colicin E1 is predominantly alpha-helical and that these helices are oriented perpendicular to the bilayer. This suggests that the bilayer-spanning segments of the channel are alpha helices. Second, both the carboxyl terminus and residue 473 have been shown to be on, or very near, the *cis* side of the bilayer in the open channel (Cleveland et al., 1983; Jakes et al., 1990). A model that incorporates these data, as well as several empirical predictions, is shown in Fig. 6 *A*. β turns predicted by the method of Paul and Rosenbusch (1985) are shown at residues 489–491 and 438–440. In addition, residues 441–458 and 420–437 are shown in Fig. 6 *C* on a helical wheel drawing. Note that both of these segments form amphipathic helices, suggesting that they may line a water-filled lumen.

In a previous paper (Jakes et al., 1990) we reported no effect on ion selectivity of removing a negative charge at residue 459 or of adding a negative charge at residue 449. Although the model for the open channel in Fig. 6 places residue 459 at the *cis* surface and residue 449 in the channel lumen, this model is not necessarily in conflict with those data, notwithstanding our previous speculation that these groups might not be near the conduction pathway. It is naive to assume that alteration of the charge state at each residue in the conduction pathway makes an equal, or even measurable, contribution to the ion selectivity. Therefore, the lack of an effect on selectivity of altering the charge of a residue does not preclude its lying in or near the conduction pathway.

The model shows residues 420–459 and 473–508 as helical hairpins in the bilayer; it is probable that regions upstream from residue 420 are also inserted in the membrane (Raymond et al., 1986), but we have no data from the present study bearing on this point. Residues 473–508 are shown as a helical hairpin, even though

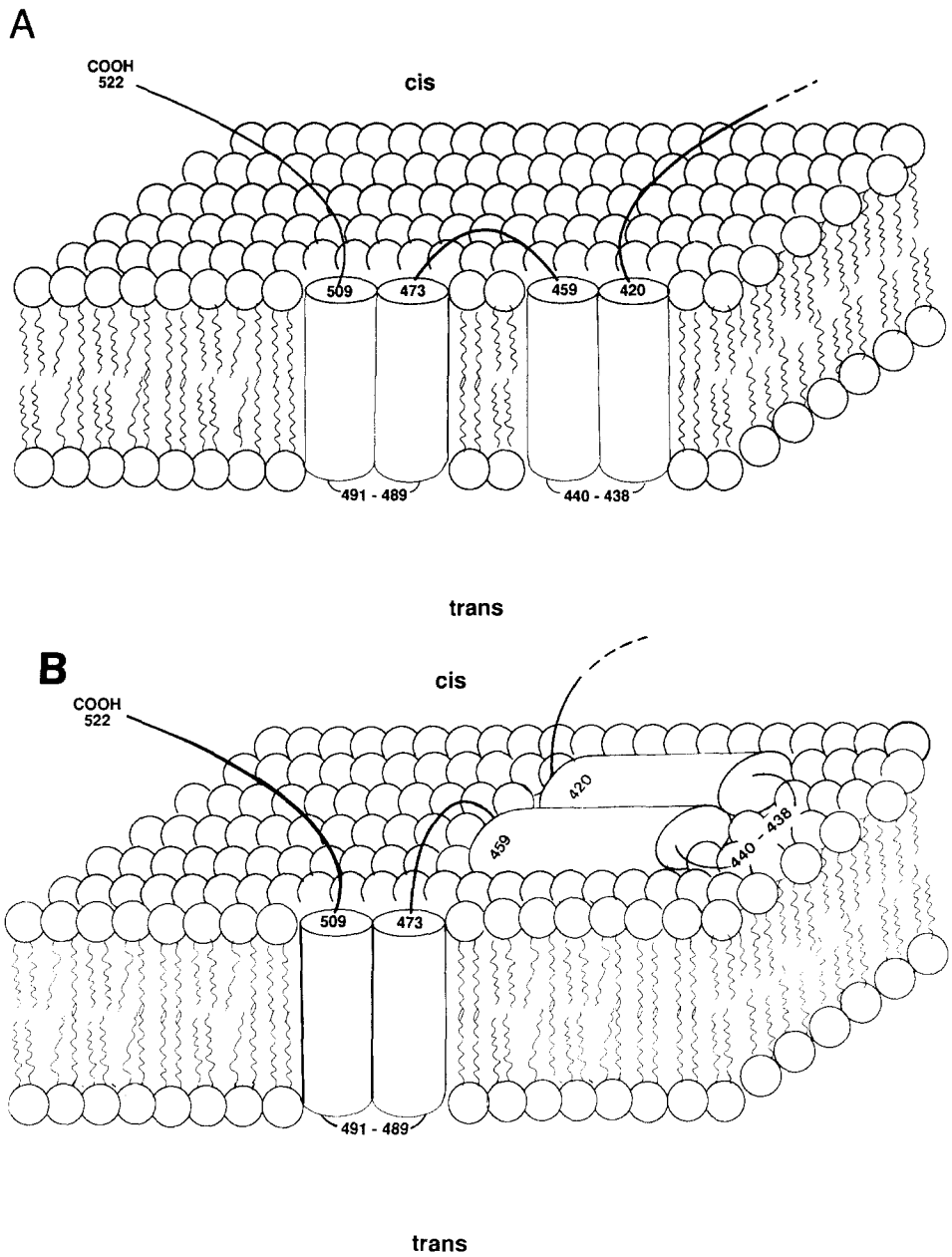


FIGURE 6. Representation of the association of colicin E1 with the membrane in the open channel (*A*) and closed channel states (*B*). The numbers refer to residue positions; cylinders represent alpha helices.

the two helices are somewhat short for spanning a lipid bilayer (16 residues, compared with the more conventional 19 or 20). However, such short, membrane-spanning helices are not unheard of (Davis and Model, 1985). Furthermore, Parker et al. (1989) recently solved the crystal structure of the channel-forming domain of a closely related protein, colicin A, which has a much longer hydrophobic segment than does colicin E1 (48 vs. 35 residues), but forms similar channels. In the crystal, at least, the hydrophobic segment of colicin A is arranged as an antiparallel pair of α helices, although without a β turn at the junction. (Interestingly, no β turn is predicted by the empirical method of Paul and Rosenbusch [1985].) E1 may require the sharp turn between the two helices, because 35 amino acids is probably a bare minimum that can span the bilayer twice; colicin A, with 48 residues, has more leeway.

2. Channel gating. The model for voltage-dependent gating is appreciated by comparing Fig. 6 *A* with Fig. 6 *B*. In this model, turn off is accomplished by the

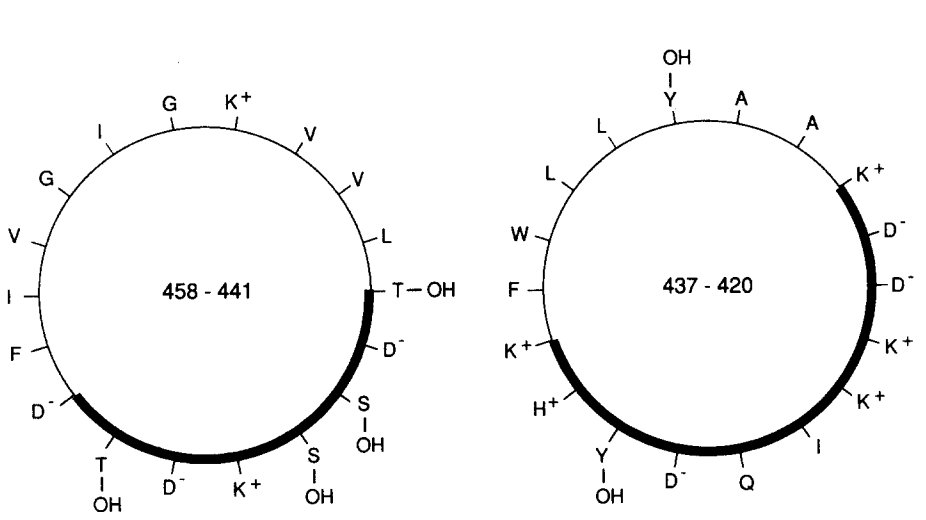


FIGURE 6. (*C*) Residues 441–458 and 420–437 are shown on helical wheels. The hydrophilic portion of these amphipathic helices is highlighted.

removal of helical hairpin 420–459 from the bilayer, where it presumably participates in the formation of an ion channel, to the lipid–water interface at the *cis* surface of the bilayer. Gating of colicin E1 channels thus involves the movement of a large segment of the protein into or out of the bilayer. This had been suggested by earlier work (Slatin et al., 1986) which showed that closed channels could be destroyed by proteases in the *cis* compartment, but open channels were protected from destruction, presumably because the protease-sensitive region of the protein was now inserted into the membrane. In addition, photolabeling experiments in vesicles with hydrophobic and hydrophilic probes (Merrill and Cramer, 1990*a*) revealed voltage-dependent changes in labeling that were consistent with insertion of protein into the bilayer upon channel opening. The gating mechanism proposed for colicin E1 channels is quite different from that proposed for several integral membrane

channels, such as the neuronal sodium channel or the nicotinic cholinergic receptor, where gating is believed to be the result of subtle movements of the protein in response to voltage or chemical ligands (Unwin, 1989); it is more consonant with the voltage-dependent gating mechanism attributed to channels formed by small peptides such as melittin (Kempf et al., 1982), or by the polyene-like antibiotic monazomycin (Heyer et al., 1976).

Note added after submission of manuscript: Upon further analysis of their photolabeling experiments in vesicles, Merrill and Cramer (1990*b*) have proposed a gating mechanism for the colicin E1 channel very similar to the one depicted in Fig. 6.

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