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Transmembrane Insertion of the Colicin Ia Hydrophobic Hairpin

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Abstract. Colicin Ia is a bactericidal protein that forms voltage-dependent, ion-conducting channels, both in the inner membrane of target bacteria and in planar bilayer membranes. Its amino acid sequence is rich in charged residues, except for a hydrophobic segment of 40 residues near the carboxyl terminus. In the crystal structure of colicin Ia and related colicins, this segment forms an α-helical hairpin. The hydrophobic segment is thought to be involved in the initial association of the colicin with the membrane and in the formation of the channel, but various orientations of the hairpin with respect to the membrane have been proposed. To address this issue, we attached biotin to a residue at the tip of the hydrophobic hairpin, and then probed its location with the biotin-binding protein streptavidin, added to one side or the other of a planar bilayer. Streptavidin added to the same side as the colicin prevented channel opening. Prior addition of streptavidin to the opposite side protected channels from this effect, and also increased the rate of channel opening; it produced these effects even before the first opening of the channels. These results suggest a model of membrane association in which the colicin first binds with the hydrophobic hairpin parallel to the membrane; next the hairpin inserts in a transmembrane orientation; and finally the channel opens. We also used streptavidin binding to obtain a stable population of colicin molecules in the membrane, suitable for the quantitative study of voltage-dependent gating. The effective gating charge thus determined is pH-independent and relatively small, compared with previous results for wild-type colicin Ia.

Key words: Streptavidin — Biotin — Channel — Voltage dependence — Bilayer

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Introduction

Colicin Ia is a water-soluble bactericidal protein that forms voltage-dependent, ion-conducting channels in the inner membrane of target bacteria, and in planar lipid bilayer membranes. It belongs to the family of channel-forming colicins (*see* reference [2] for a review), whose members each consist of three domains, responsible for (i) binding to a receptor in the outer membrane (middle domain), (ii) translocation to the inner membrane (N-terminal domain), and (iii) channel formation (C-terminal domain). Near the carboxyl end of the channel-forming domain is a hydrophobic segment, which is present in all the channel-forming colicins; in colicin Ia it is 40 residues long (residues 573–612) [12]. The hydrophobic segment forms an α-helical hairpin in the crystal structures of colicins A, E1, and Ia [18, 24, 25]. It is thought that this helical hairpin structure is preserved when the colicin changes its conformation to form a transmembrane channel.

The role of the hydrophobic segment in the initial interaction of the colicin with the membrane and in the subsequent formation of a channel remains unsettled. Numerous studies of colicins E1, A, and Ia (or their C-terminal domains) have led to the conclusion that the hydrophobic segment inserts into the membrane when the channel is closed [1, 9, 11, 14, 17, 18, 21, 27]; it has been suggested that this insertion might be the initial step in the entry of the colicin into the membrane. In addition, there is evidence that the hydrophobic segment must reach across the membrane as a hairpin for the conducting channel to be formed [23]. Recent experiments with colicin A, however, have indicated that the hydrophobic hairpin lies near the surface of the membrane when the channel is closed [3, 4, 6, 10, 13]; it should be noted, though, that the phosphatidylglycerol membranes used in those studies do not support channel formation, at least not by colicin E1 [28].

We have previously mapped the locations of a number of residues of the colicin Ia channel-forming domain with respect to a planar bilayer membrane, by using the streptavidin-biotin system [19]. In this method, a biotin group is covalently attached to a specific residue of the colicin, to create a potential binding site for streptavidin. After the membrane is exposed to the biotinylated colicin, streptavidin is added to the solution bathing one side of the membrane. If streptavidin addition alters the electrical properties of the colicin channels, it indicates that the biotin group on the colicin is accessible to the streptavidin, and hence exposed to the aqueous solution on that side of the membrane. Using this technique, we have shown that a region of at least 68 residues of colicin Ia is translocated back and forth across the membrane in conjunction with the voltage-dependent opening and closing of the channels [20, 22].

We have now used the streptavidin-biotin system to examine the orientation of the hydrophobic hairpin of colicin Ia, by probing the location of a residue at the tip of the hairpin. This work has appeared in a preliminary form [8].

Materials and Methods

Mutagenesis and Biotinylation of Colicin Ia

A unique cysteine was introduced at residue 594 of colicin Ia, in the middle of the hydrophobic segment [12], by site-directed oligonucleotide mutagenesis, using the mutagenic oligonucleotide 5′-CTTAC-CGGATGCGCTTTAG-3′. Mutagenesis and the expression and purification of the mutant protein were performed as described in reference [19]. A biotin group was covalently attached to the cysteine residue as previously described [20], using one of the following sulfhydryl-specific reagents: N-[6-(biotinamido)hexyl]-3′-(2′-pyridyldithio)propionamide (biotin-HPDP), N-[2-(biotinamido)ethyl]-3′-(2′-pyridyldithio)propionamide (biotin-EPDP) (both from Pierce Chemical, Rockford, IL), or N-(biotinoyl)-N′-(iodoacetyl)ethylenediamine (Molecular Probes, Eugene, OR). Note that colicin that has reacted with biotin-HPDP or biotin-EPDP can subsequently be reduced to regenerate the original, unbiotinylated protein. The biotinylated colicin S594C was then usually purified on a monomeric avidin column (Pierce Chemical) as described [20]. Stock solutions of wild-type colicin Ia and the cysteine mutant S594C were stored frozen at −70°C in 300 mM NaCl, 25 or 50 mM sodium borate, 2 mM EDTA, 2 mM dithiothreitol, pH 9.0, at concentrations >1 mg/ml; thawed aliquots and biotinylated mutant were kept at 4°C, where they generally retained channel-forming activity for months. Streptavidin was obtained from Calbiochem Corp. (La Jolla, CA).

Planar Bilayer Experiments

Membranes were formed at room temperature from asolectin (lecithin type IIS; Sigma Chemical, St. Louis, MO) from which nonpolar lipids had been removed [7]. Lipid (1% in pentane) was layered on top of the aqueous solutions in the two compartments on either side of a Teflon partition. The partition contained an 80–130 μm hole which was pretreated with 3% squalene in petroleum ether. After the solvents evaporated, the lipid layers were raised over the level of the hole, forming the membrane [15]. The volume of solution on each side of the membrane was ~1 ml and contained (in mM): 100 KCl, 5 $CaCl_2$, 1 EDTA, and 20 of an appropriate buffer: MES at pH 6.2, HEPES at pH 8.0, or CHES at pH 9.0. The transmembrane voltage (V) was clamped, and the resulting current (I) was monitored as previously described [5]; the membrane conductance, g, is I/V. All voltages are those of the *cis* compartment, defined as the side to which colicin was added, with respect to that of the opposite *trans* compartment. Both compartments could be stirred by small magnetic stir bars. Membrane capacitance was frequently checked during experiments, to verify that the membrane area had not drastically changed. Colicin (generally 1–2 mg/ml) was usually diluted 1:1 (vol:vol) with 1% octylglucoside (Calbiochem) before the addition of ~1 μl to the *cis* compartment; dilution with octylglucoside yielded more channels per milligram of protein without otherwise affecting channel behavior. In single-channel experiments, the KCl concentration was 1 M, and octylglucoside was omitted. In specified experiments, colicin was removed from the *cis* solution by perfusing for 1–2 min with 10 bath volumes of fresh solution.

After the conductance induced by the biotinylated colicin S594C was stabilized by the addition of streptavidin to the *trans* and *cis* solutions (*see below*), steady-state conductance-voltage relations could be determined. The conductance was turned on by stepping the voltage across the membrane from zero to a *cis* positive value, which was maintained until the conductance stopped increasing. A steady-state value was generally obtained within 10–30 sec, although near V_0, the voltage producing a half-maximal conductance, it could take 1–5 min. (Stepping the voltage from a larger to a smaller positive value generally gave a comparable steady-state conductance.) The conductance was then turned off by a step to a *cis* negative voltage; in the subsequent analysis, any residual conductance at this point was subtracted as a nonspecific leak. To determine the effective gating charge, n, the data were fit by the formula

$$\ln[g/(g_{max} - g)] = n(V-V_0)F/RT, \quad (1)$$

where g_{max} is the maximal conductance, and F, R, and T are the Faraday constant, gas constant, and absolute temperature, respectively [16]. When g_{max} could not be accurately determined, the gating charge was obtained by plotting ln g against V, and setting the slope of the curve between 20 and 40 mV equal to nF/RT.

Results

Trans Streptavidin Alters Channel Kinetics

When added to the *cis* solution, the biotinylated colicin Ia mutant S594C induced a normal voltage-dependent conductance across a planar bilayer, with channels opening at *cis* positive voltage and closing at *cis* negative voltage. At pH 8.0 and 9.0, the rate of channel opening was fairly slow. After streptavidin was added to the opposite *trans* solution, the channels began to open more quickly in response to a positive voltage stimulus; in addition, the current became progressively noisier with time (Fig. 1). These effects were not observed if an excess concentration of free biotin was added to the *trans* solution before the streptavidin, in order to block the biotin-binding sites of the streptavidin; neither did *trans* streptavidin affect unbiotinylated S594C or wild-type colicin Ia channels.

At pH 6.2, the rate of channel opening was relatively fast before streptavidin addition, both for biotinylated

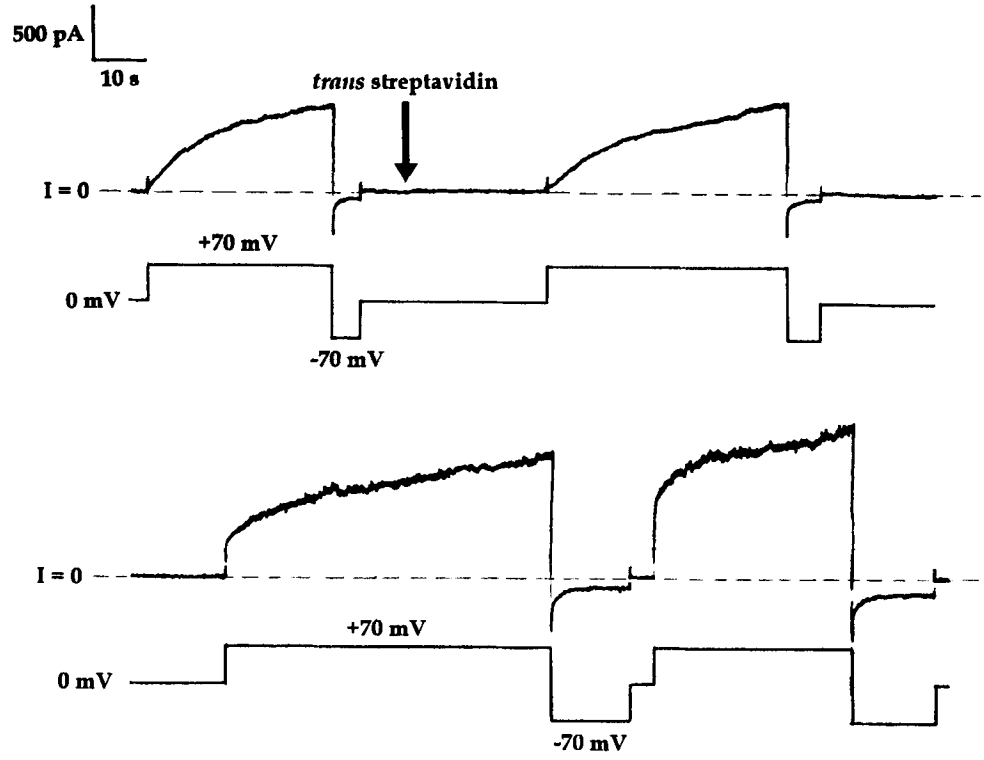

Fig. 1. The effect of *trans* streptavidin on the conductance induced by the biotinylated mutant colicin S594C at pH 9.0. Several minutes before the start of the record, 2.0 μg of biotinylated S594C (plus 15 μg octylglucoside) were added to the *cis* compartment, and the membrane was pulsed several times between ±50 or ±70 mV to confirm normal voltage gating. The record begins with one such ±70 mV sequence, showing the slow rate of turn-on of the current at +70 mV, and the relatively low current noise. After this pulse, 10 μg of streptavidin were added to the *trans* compartment. There was not much effect on the current in the next pulse, but in the two subsequent pulses, the current turned on much more quickly in response to a step to +70 mV, and the current at this voltage became noisier. (The lower current and voltage traces are uninterrupted continuations of the upper traces.) The fact that *trans* streptavidin had an effect on the current response indicates that the biotin group at residue 594 was exposed to the *trans* solution. Solutions on both sides of the membrane were (in mM): 100 KCl, 5 $CaCl_2$, 1 EDTA, 20 CHES, pH 9.0. The current was low-pass filtered at 100 Hz. The colicin was biotinylated with biotin-HPDP and purified on a monomeric avidin column.

S594C and for wild-type colicin Ia. In some experiments with biotinylated S594C, *trans* streptavidin increased the opening rate, as at higher pHs. In other experiments, in which the opening rate was initially very fast, the most prominent effect of *trans* streptavidin was to increase the conductance (Fig. 2). In both cases, the current noise increased after *trans* streptavidin addition.

Cis Perfusion and Cis Streptavidin Each Reduce Channel Activity

Before we describe another effect of *trans* streptavidin on colicin behavior, it is necessary to mention how perfusion of the *cis* solution affected the colicin conductance. At pH 8.0, following perfusion of the *cis* side with fresh bath solution, the colicin-induced conductance gradually became smaller in successive voltage pulses (Fig. 3*A*), typically falling by 90% in about 10 min. The rate of decline was much faster from the closed state than from the open state. This indicates that at this pH, after the channels closed, the colicin could dissociate from the membrane into the *cis* solution at an appreciable rate. With the *cis* solution depleted of colicin by perfusion, there would be no subsequent binding of colicin to the membrane.

Streptavidin added to the *cis* solution produced an effect similar to that of *cis* perfusion. In part, this is because colicin in solution which binds streptavidin at residue 594 is unable to form channels, as shown by preincubation experiments with streptavidin (*data not shown*); hence, like *cis* perfusion, *cis* streptavidin removes active colicin from the solution. *Cis* streptavidin actually reduced the conductance more rapidly than did *cis* perfusion. If streptavidin was stirred into the *cis* solution while the channels were held open (e.g., at +50 mV), the conductance in subsequent voltage pulses typically dropped by 99% within 1–2 min (*data not shown*). This enhanced rate of conductance decline indicates that *cis* streptavidin also interacts directly with membrane-bound colicin.

At lower pH (6.2), the effect of *cis* streptavidin developed more slowly, suggesting that residue 594 is less accessible to the *cis* side at this pH.

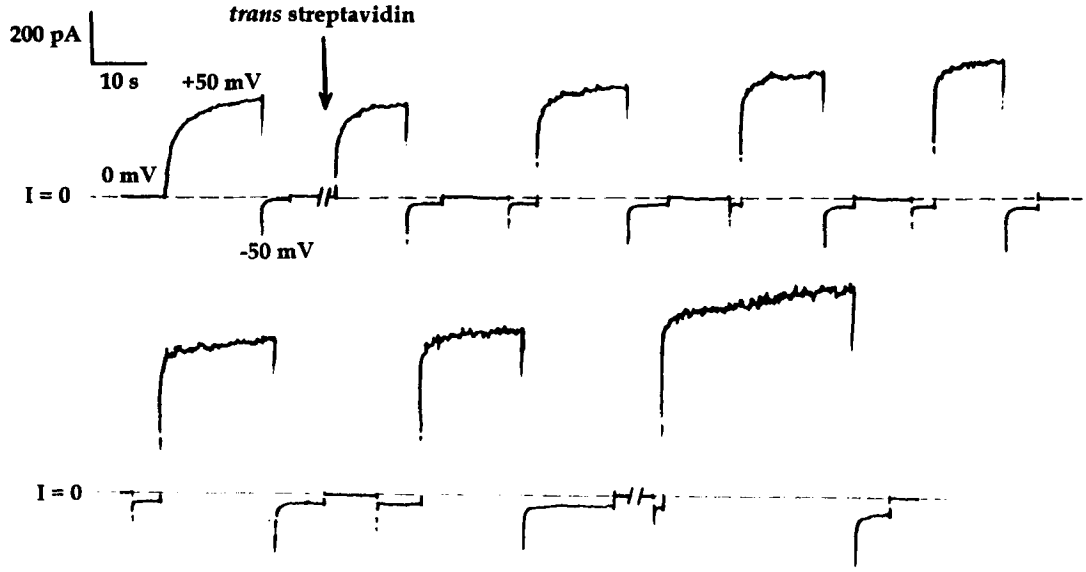

Fig. 2. The effect of *trans* streptavidin on the conductance induced by biotinylated S594C at pH 6.2. Several minutes before the start of the record, 0.3 μg of biotinylated S594C (plus 3.5 μg octylglucoside) were added to the *cis* compartment, and the membrane was pulsed several times between ±50 mV to confirm normal gating. The magnitude of the conductance became fairly stable 2–3 min before the start of the record. The record begins with a ±50 mV sequence, showing the fast turn-on of the current at +50 mV. During the first break in the record (1 min), 40 μg of streptavidin were added to the *trans* compartment. Over the next few minutes, the conductance at +50 mV gradually increased, doubling by the end of the record; in addition, the current noise increased, and the turn-on of the current became even faster than it was initially. The lower current trace is an uninterrupted continuation of the upper trace. Solutions on both sides of the membrane were (in mM): 100 KCl, 5 $CaCl_2$, 1 EDTA, 20 MES, pH 6.2. The biotinylation of S594C was with N-(biotinoyl)-N′-(iodoacetyl)ethylenediamine. The second break in the record was 45 sec.

Trans Streptavidin Anchors Biotinylated S594C to the Membrane

In addition to its other effects, streptavidin added to the *trans* solution prevented the washout of colicin activity normally caused by perfusion of the *cis* solution. This is shown in Fig. 3*B*, which begins several minutes after the addition of streptavidin to the *trans* solution. Four minutes after perfusion of the *cis* side with fresh bath solution (first break), there was little decrease in the conductance, and the remaining conductance could be stable for hours. The three effects of *trans* streptavidin—fast turn-on, increased noise, and prevention of washout—show that the biotin group attached to residue 594 is exposed to the *trans* solution.

Trans streptavidin also blocked the effect of *cis* streptavidin, preventing the reduction in colicin activity that it would otherwise have caused (*data not shown*). The anchoring effect of *trans* streptavidin was prevented by the prior addition of excess free biotin to the *trans* solution.

For colicins biotinylated with a reducible reagent, we found that not only was the biotin exposed to the *trans* side, so was the disulfide bond linking it to the colicin. Following the *trans* streptavidin addition and *cis*-side perfusion described above, free cysteine, a membrane-impermeant reducing agent, was added to the *trans* solution to reduce the disulfide bond and free the bound streptavidin from the colicin (Fig. 3*B*, second break). Subsequently, the conductance decreased gradually over several minutes. Thus, *trans* cysteine abolished the anchoring effect of *trans* streptavidin, so that the colicin was finally able to dissociate from the membrane. In experiments using *cis* streptavidin in place of *cis* perfusion, *trans* cysteine produced a similar reversal of anchoring.

Trans Streptavidin Effects on Single Channels

The anchoring and kinetic effects induced by *trans* streptavidin in many-channel membranes were also observed on single channels of biotinylated colicin S594C (Fig. 4). In the absence of streptavidin, these channels resembled wild-type channels. Before streptavidin addition, there was generally a long delay after a positive voltage step before a channel opened. Typically 5–10 min after streptavidin was added to the *trans* solution, however, the channel began to open very quickly, within a fraction of a second, in response to a positive voltage step. The single-channel conductance was not affected. The subsequent addition of *cis* streptavidin had no effect on channel activity in over 30 min, whereas in experiments without *trans* streptavidin, *cis* streptavidin would eliminate channel activity within a few minutes. Thus *trans* streptavidin blocked the effect of *cis* streptavidin. Finally, free cysteine added to the *trans* solution caused the channel to disappear after a few minutes, indicating that

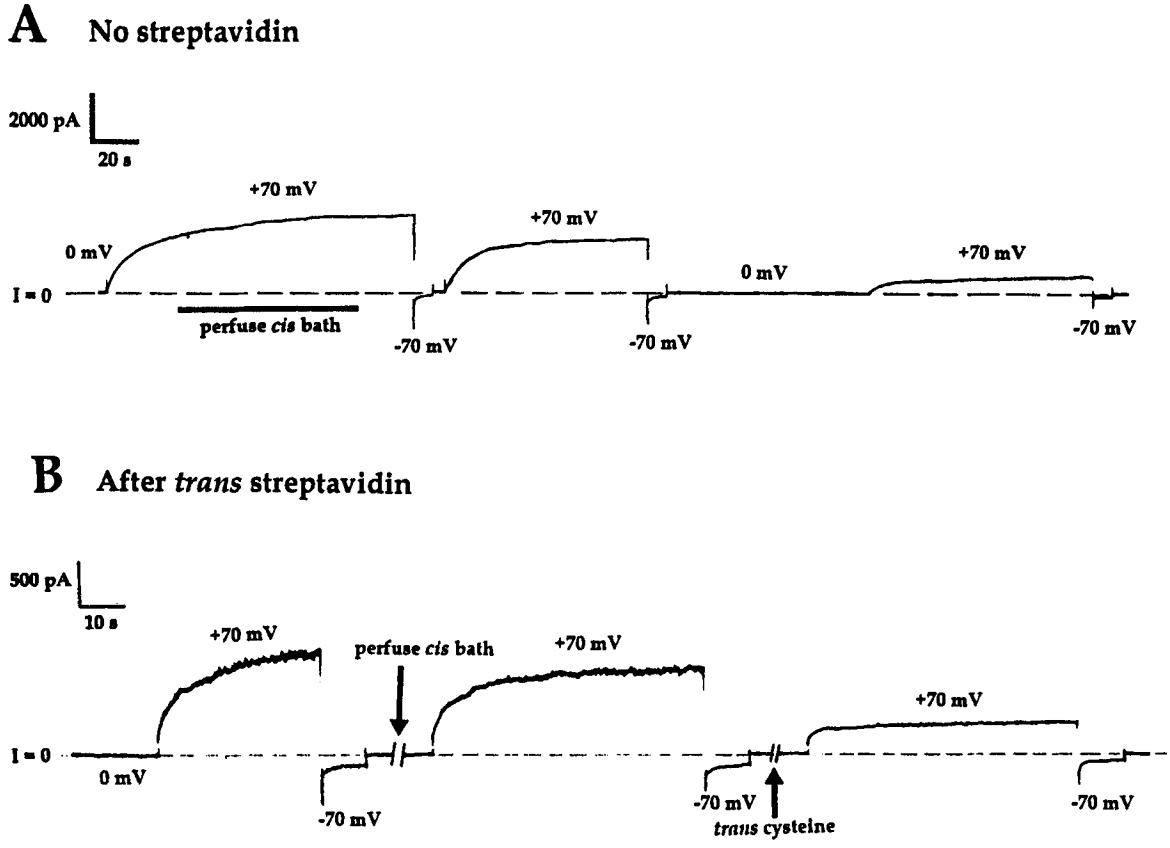

Fig. 3. The effect of *cis* perfusion on the conductance induced by biotinylated S594C, with and without *trans* streptavidin. (*A*) The effect of *cis* perfusion alone. Several minutes before the start of the record, 2.0 μg of biotinylated S594C (plus 15 μg octylglucoside) were added to the *cis* compartment, and the membrane was pulsed several times between ±50 or ±70 mV to confirm normal gating. In the record, with the voltage held at +70 mV and the colicin-induced current rising, the *cis* compartment was perfused with 10 volumes of fresh bath solution (solid bar). After this perfusion, the current stopped increasing. Following a brief pulse to −70 mV to turn off the current, the next pulse to +70 mV showed a slightly smaller current. After a longer period of turn-off, the following pulse to +70 mV showed a substantially reduced current; the current continued to decline in subsequent pulses for several minutes after the end of the record. This shows that the colicin could dissociate from the membrane. (*B*) The effect of *cis* perfusion after *trans* streptavidin addition. Several minutes before the start of the record, 2.0 μg of biotinylated S594C (plus 15 μg octylglucoside) were added to the *cis* compartment, and the voltage was pulsed to confirm normal gating. Then 10 μg of streptavidin were added to the *trans* compartment. The record begins several minutes later with a ±70 mV sequence, after the development of an increase in the current noise and turn-on rate. After the current was turned off at −70 mV, the *cis* compartment was perfused (at $V = 0$ mV) with 10 ml of fresh bath solution (at the beginning of the first break, 5 min). In contrast to the results of panel *A*, here the current at +70 mV remained stable, at a slightly reduced level. Thus, *trans* streptavidin bound to biotinylated S594C anchored the colicin to the membrane. During the second break in the record, cysteine dissolved in buffer solution was added to the *trans* compartment to a concentration of 15 mM, to reduce the disulfide bond linking the streptavidin-biotin complex to the colicin. Subsequently, as shown after the break, the current at +70 mV decreased substantially (this pulse occurred 4 min after cysteine addition); it continued to decline for several minutes after this. Thus, after the bound *trans* streptavidin was freed from the colicin, the colicin could dissociate from the membrane. For both panels, the solutions on both sides of the membrane were (in mM): 100 KCl, 5 $CaCl_2$, 1 EDTA, 20 HEPES, pH 8.0. The colicin was biotinylated with biotin-HPDP and purified on a monomeric avidin column. The second break was 7 min long.

trans cysteine had reversed the anchoring effect of *trans* streptavidin. These results at the single-channel level are completely consistent with the macroscopic data described above. We did not, however, observe changes in single-channel currents corresponding to the increased noise seen in the macroscopic current after *trans* streptavidin addition. This could indicate that the increased noise depends on an interaction among many channels; alternatively, it could be that only a small fraction of the channel population contributes to the noise.

Trans Streptavidin Acts on Channels That Have Never Been Opened

The effects of *trans* streptavidin described above indicate that the colicin Ia hydrophobic hairpin reaches across the membrane at some point during channel formation. To ascertain whether biotinylated residue 594 was accessible to *trans* streptavidin when the channels were closed, and, in particular, before the channels had ever been opened, the membrane was held at a potential of

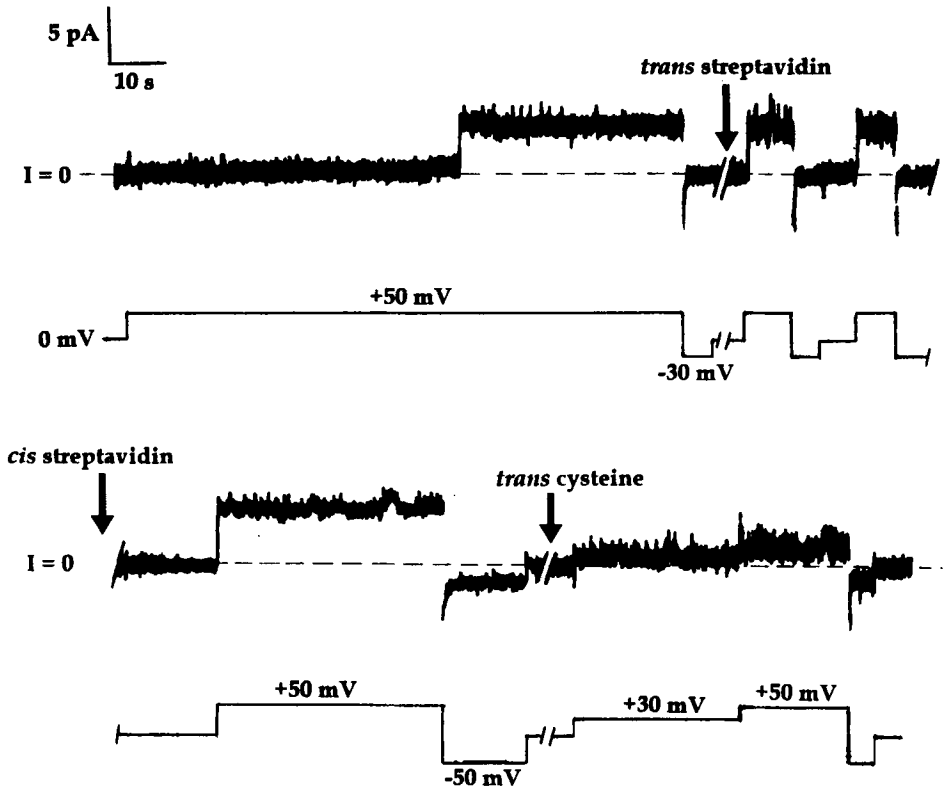

Fig. 4. The effect of *trans* streptavidin on biotinylated S594C at the single-channel level. Several minutes before the start of the record, 26 μg of biotinylated S594C were added to the *cis* compartment. As shown at the beginning of the record, a single channel was opened by a step to +50 mV, with a delay of about 1 min before opening; it closed quickly in response to a negative voltage step. At the beginning of the first break in the record (5 min), 10 μg of streptavidin were added to the *trans* compartment. Following this, the channel began to open very quickly in response to a pulse to +50 mV. At the beginning of the second break (33 min), 10 μg of streptavidin were added to the *cis* compartment; *cis* streptavidin had no apparent effect on the channel in this interval. During the last break in the record (12 min), cysteine in buffer solution was added to the *trans* compartment to a concentration of 15 mM; after 5 min, the channel ceased to open in response to positive voltage pulses. These results on single channels are in harmony with the anchoring and kinetic effects of *trans* streptavidin on the macroscopic conductance. Solutions on both sides of the membrane were 1 M KCl, 5 mM $CaCl_2$, 1 mM EDTA, 20 mM CHES, pH 9.0. The colicin was biotinylated with biotin-HPDP and purified on a monomeric avidin column.

−50 mV, to prevent the channels from opening, and biotinylated colicin S594C was added to the *cis* solution (Fig. 5). Streptavidin was then added to the *trans* solution, and after a 5-min exposure, the unbound *trans* streptavidin was inactivated by adding excess free biotin to the *trans* solution. Thus, only when the channels were closed were they exposed to active *trans* streptavidin. Streptavidin was then also added to the *cis* solution, so that colicin which had not bound *trans* streptavidin would be prevented from forming channels. After all these maneuvers, colicin channels were still able to open in response to a positive voltage step, with the fast turn-on and increased noise characteristic of *trans* streptavidin-bound channels. In control experiments, with *trans* biotin added before *trans* streptavidin, there was no channel activity at this point. Thus, the tip of the hydrophobic hairpin was exposed to the *trans* solution before the channels had ever been opened, at least for some fraction of the colicin molecules.

Given these effects of *trans* streptavidin on closed channels, we expected that it could also act on open channels. We tested this by opening channels with a positive voltage step, adding *cis* streptavidin to prevent new channels from entering the membrane, and then adding *trans* streptavidin for a 5-min exposure, followed by *trans* biotin, before closing the channels. The subsequent colicin-induced conductance showed the characteristic effects of *trans* streptavidin binding: fast turn-on, increased noise, and stable activity (*data not shown*). This confirmed that the tip of the hydrophobic hairpin was accessible to *trans* streptavidin when the channels were open.

Relative Rates of Cis and Trans Streptavidin Effects on Closed Channels

At high pH, the tip of the inserted hydrophobic hairpin can move back to the *cis* side, as indicated by the reduc-

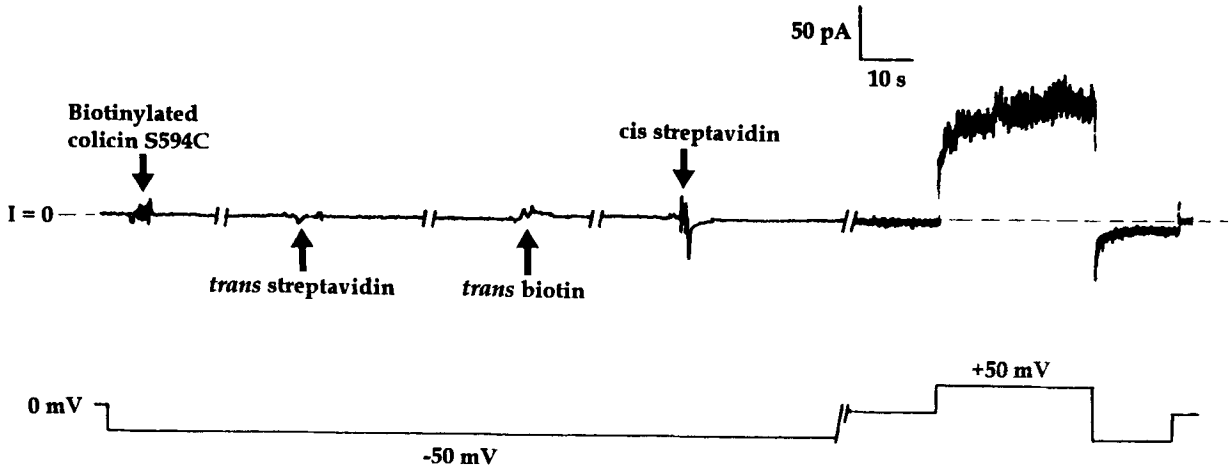

Fig. 5. The effect of *trans* streptavidin on the conductance induced by biotinylated S594C, before the conductance had ever been turned on. The record begins before colicin addition, with the membrane potential held at −50 mV to prevent channel opening. At the first arrow, 2.0 μg of biotinylated S594C (plus 15 μg octylglucoside) were added to the *cis* compartment. Next, 40 μg of streptavidin, followed after 5 min by 5 μg of biotin (a 30-fold molar excess), were added to the *trans* compartment; this ensured that only closed channels were exposed to active *trans* streptavidin. Then 10 μg of streptavidin were added to the *cis* compartment, to prevent any further entry of colicin into the membrane. When the voltage was now stepped to +50 mV, the conductance was still able to turn on, with the kinetics and noise characteristic of biotinylated S594C bound to *trans* streptavidin. This shows that the hydrophobic hairpin can insert across the membrane before channel formation. Solutions, biotinylation and purification were as in Fig. 1. The breaks in the record were, sequentially, 80 sec, 4.5 min, 40 sec, and 85 sec.

tion in conductance caused by *cis* perfusion and *cis* streptavidin. Thus, while the channel is closed, there is an equilibrium between states with residue 594 on the *cis* or *trans* side. To assess the relative exposure of this residue on either side of the membrane, we allowed *cis* and *trans* streptavidin to compete for the biotinylated colicin. After colicin was added to the *cis* solution, the voltage was pulsed several times to turn the channels on and off, establishing the level of activity. While the channels were closed, and the voltage was held at 0 mV, streptavidin was added simultaneously and in equal concentration to the *cis* and *trans* solutions; upon testing after a 10-min exposure, the colicin activity was almost completely eliminated (at pH 9.0) (*data not shown*). In contrast, if *trans* streptavidin was added 10 min before *cis* streptavidin, most of the activity was preserved. Intermediate times produced intermediate results. Thus, it appears that for closed channels at pH 9.0, the tip of the hydrophobic hairpin spent more time on the *cis* side than on the *trans* side. (It is also possible that the difference in rates reflects greater steric hindrance to the binding of *trans* streptavidin, compared with *cis* streptavidin.) Essentially all the colicin molecules, however, had the tip of the hydrophobic hairpin exposed to the *trans* side at some point during a 10-min interval.

When similar experiments were done at pH 6.2, with streptavidin added simultaneously to both sides of the membrane, the colicin conductance decreased to 25–30% of its initial value. Thus, *cis* streptavidin still acted more quickly than *trans* streptavidin at this pH, but the difference in rates was not as great as at pH 9.0. This result indicates that for closed channels the hairpin spends more time inserted across the membrane at lower pH. This is consistent with the slower development of the *cis* streptavidin effect at lower pH, mentioned above.

Voltage-Dependent Gating of Anchored Channels

It has been difficult in the past to study colicin channel gating quantitatively, due to the complexity of the gating; the number of active channels in a membrane tends to vary over the course of an experiment, even with a fixed voltage protocol. By using *trans* streptavidin to anchor the hydrophobic hairpin across the membrane, we have produced a more stable channel-membrane system. Thus anchored, the colicin can no longer dissociate from the membrane. The subsequent addition of *cis* streptavidin prevents new colicin from entering the membrane to form channels. The result is a stable population of colicin channels in the membrane, which should be more amenable to quantitative study than is the standard colicin.

As an illustration of the advantages of this system, we have measured the voltage dependence of channel gating for the anchored colicin, as reflected by the steady-state conductance-voltage relation. A previous study of the (unanchored) wild-type colicin Ia channel found that the voltage dependence of the conductance (or, more precisely, of the component that turned on rapidly in response to a voltage step) depended on the pH, with an effective gating charge ranging from 3.1 at

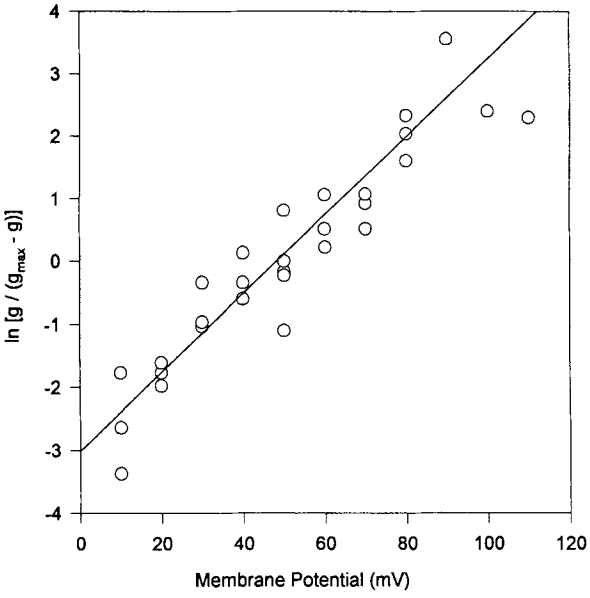

Fig. 6. Steady-state conductance-voltage relation for biotinylated S594C, anchored to the membrane by *trans* streptavidin. The experiment began with the addition of 2.0 μg of biotinylated S594C (with 15 μg octylglucoside) to the *cis* compartment, followed by the addition of 10 μg of streptavidin to the *trans* compartment. The voltage was pulsed until the fast turn-on of the current had developed sufficiently (about 13 min). Streptavidin (10 μg) was then added to the *cis* compartment, and the current was held off at −80 mV for 2-min intervals until the current at +80 mV stabilized (about 5 min later). Over the next 100 min, steady-state currents were measured by pulsing from 0 mV to the test voltage, and also, for test voltages ≤50 mV, by pulsing from a larger voltage. To determine the leak current for each pulse, the current was turned off at −50 mV, or at a more negative potential. Steady-state and leak currents were estimated by eye from the chart record. The graph shows the leak-subtracted steady-state conductance, g, transformed as $\ln[g/(g_{max} - g)]$ (open circles), plotted against the test voltage; the maximal conductance was set to g_{max} = 2400 pS. Fitting with Eq. 1 (straight line) provided the gating charge, n = 1.6, and the voltage producing a half-maximal conductance, V_0 = 48 mV. Solutions, biotinylation and purification were as in Fig. 1.

pH 8.2 to 6.2 at pH 4.5 [16]. For the streptavidin-anchored colicin, we found that the conductance-voltage data at pH 9.0 were well fit by Eq. 1, with effective gating charge $n = 1.7 \pm 0.2$ (8 experiments) and voltage midpoint $V_0 = 54 \pm 7$ mV (3 experiments) (*e.g.*, Fig. 6). This gating charge is smaller than the value ($n \sim 2.5$ to 3.0 at pH 9.0) obtained by extrapolation from the wild-type data of reference [16].

To measure conductance-voltage relations at pH 6.2, most of the experiments were begun at pH 9.0 to ensure that colicin in the membrane which did not bind *trans* streptavidin could be effectively removed by *cis* streptavidin, as discussed above. (Essentially the same results were obtained if the entire experiment was done at pH 6.2.) After the conductance was stabilized by *trans* and *cis* streptavidin at pH 9.0, the bath solutions were acidified to pH 6.2 by the addition of concentrated MES buffer. The gating charge measured at pH 6.2 for the streptavidin-anchored colicin, $n = 1.8 \pm 0.2$ (5 experiments), and the voltage midpoint, $V_0 = 42 \pm 5$ mV (3 experiments), were not significantly different from their values at pH 9.0. The gating charge was found to be independent of pH even when conductance-voltage curves were measured at both pH 9.0 and 6.2 on the same membrane. At pH 6.2, the discrepancy with the wild-type result, n = 5.3 [16], was even greater than at pH 9.0.

Discussion

We have used the interaction of streptavidin with the biotinylated mutant colicin S594C to study the conformation of the hydrophobic hairpin in planar bilayer membranes. Our results can be explained by the minimal model in Fig. 7. (This model is similar to Fig. 4 of reference [2].) First, we have shown that the channel can open and close with the tip of the hairpin held on the *trans* side, as in states 3 and 4; this transition corresponds to the voltage-dependent insertion and de-insertion of the previously identified translocated region [20, 22]. Second, after colicin is added to the *cis* solution (state 1), it can first bind to the membrane with the tip of the hairpin on the *cis* side (state 2), and then the tip can get to the *trans* side of the membrane (state 3), even with a large negative voltage (−50 mV) preventing channel formation. Finally, the tip of the hairpin can return from the *trans* side (state 3) back to the *cis* side (state 2), apparently spending more time on the *cis* side than the *trans* side when the membrane potential is zero; the colicin can also wash out of the membrane, from state 2 back to state 1.

Our results may clarify some of the unsettled issues about the orientation of the hydrophobic hairpin. According to the model in Fig. 7, the hydrophobic hairpin is parallel to the membrane surface at an early stage of binding (state 2), but it is inserted across the membrane at a later stage (state 3). This insertion can occur without channel opening, and with a negative transmembrane potential. (This shows that the insertion cannot be strongly voltage-dependent.) Several experiments on colicin A failed to detect any colicin in state 3 [3, 4, 6, 10, 13]. It could be that the methods used were not sensitive enough to detect a small fraction of the colicin molecules in state 3; alternatively, the colicin may have been arrested in state 2, due to the high lipid charge density in those experiments [28]. Despite the unphysiological lipid composition in those experiments, our results on colicin Ia support the conclusion that some of the colicin molecules have their hydrophobic hairpins oriented parallel to the membrane. The many previous studies supporting a transmembrane orientation for the hydrophobic hairpin in the closed state [9, 11, 14, 17, 21] would indicate a high occupancy of state 3. This is not incon-

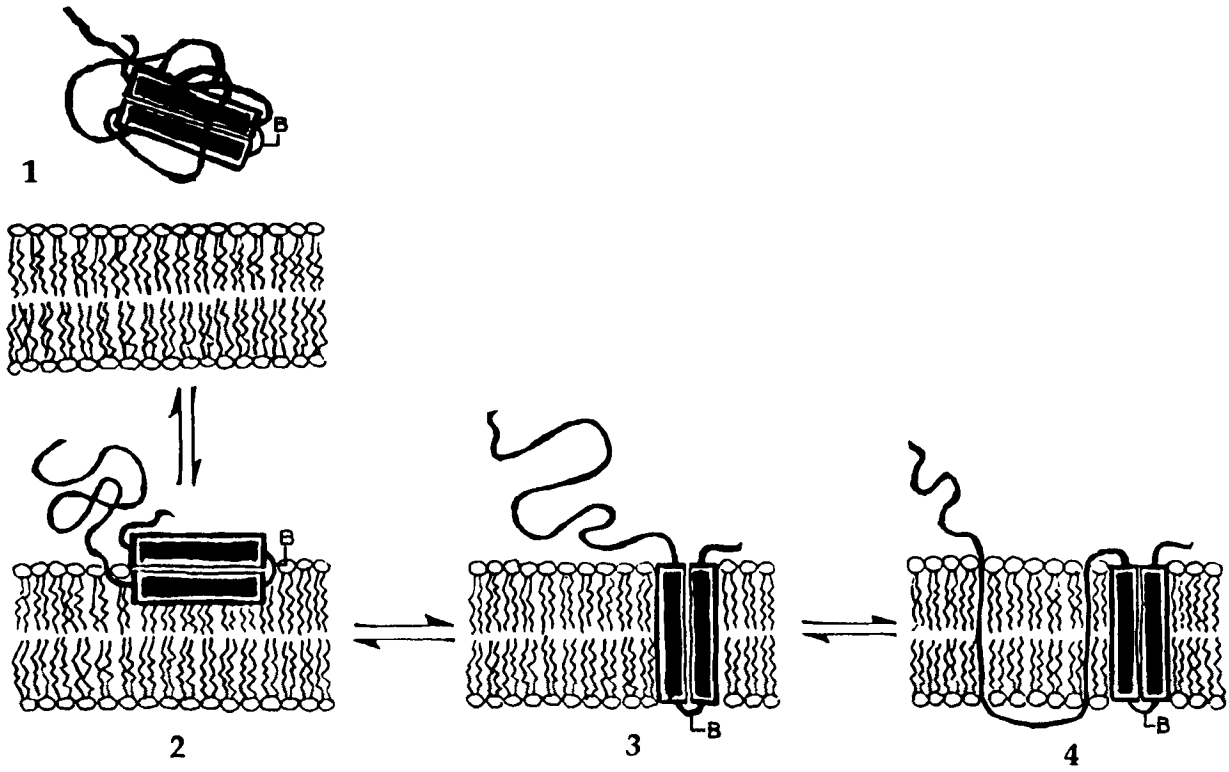

Fig. 7. Model of the orientation of the colicin Ia hydrophobic hairpin with respect to the membrane. The hydrophobic hairpin is represented as two bars connected by a loop, with a biotin group (*B*) attached to the loop at residue 594; the rest of the molecule is shown schematically as a wiggly line. (1) Colicin in the *cis* solution. (2) Colicin bound to the membrane, with the tip of the hairpin exposed to the *cis* solution. (3) Colicin with the hairpin inserted into the membrane and the tip exposed to the *trans* solution. States 1, 2, and 3 are nonconducting. (4) Colicin with the hairpin inserted as in state 3, and with an additional segment translocated across the membrane to complete the formation of the open channel. Only the transition between states 3 and 4 is significantly voltage dependent, and each of the state transitions is reversible.

sistent with our results: although we found state 2 to be favored over state 3, the hairpin spent more time inserted at pH 6.2 than at pH 9.0. The previous experiments were generally done at even lower pH (4.1 in the colicin Ia experiments of reference [14]), where an even greater degree of insertion might be expected.

Although some aspects of our model for colicin Ia could apply to other colicins, there are likely to be areas of difference. For instance, the binding of colicin E1 (or its channel-forming domain) to lipid vesicles at pH 4 is more or less irreversible, even when the pH is subsequently raised to 8 [28], whereas our experiments indicate that colicin Ia can dissociate from a planar bilayer at pH 8. (At high ionic strength, however, the colicin E1 channel-forming domain shows significant dissociation from vesicles even at pH 6 [26].) In addition, given that colicin hydrophobic segments range in length from 35 to 49 residues [2], it is likely that different colicins strike different balances between states 2 and 3. It is also possible that the biotin group attached to the colicin in our experiments could have some influence on the orientation of the hairpin.

In the context of our model, we can suggest some possible mechanisms for the streptavidin effects on biotinylated colicin S594C. Preincubation of the colicin with streptavidin, or binding to *cis* streptavidin, holds the tip of the hydrophobic hairpin on the *cis* side of the membrane (states 1 and 2), so the colicin cannot form an open channel (state 4). (We assume that the large, hydrophilic streptavidin molecule cannot be moved across the membrane.) Conversely, binding to *trans* streptavidin holds the tip of the hairpin on the *trans* side (states 3 and 4), so that the colicin-induced conductance is not diminished by perfusion of the *cis* bath (which depletes state 1), or by *cis* streptavidin (which acts on states 1 and 2). The fast turn-on induced by *trans* streptavidin reflects the voltage-dependent transition from state 3 to state 4; presumably, without streptavidin bound, slower transitions limit the rate of channel opening. From the relative rates at which the *cis* and *trans* streptavidin effects develop when the channels are closed, it appears that state 2 is favored over state 3 at pH 9.0; therefore, the transition from state 2 to state 3 could be partly responsible for the slow rate of channel opening at this pH. At pH 6.2, the opening rate is faster than at pH 9.0, consistent with the increased occupancy of state 3 at the

lower pH. An explanation of the increased noise caused by *trans* streptavidin would probably require a more complex model than the one in Fig. 7; without a clear effect on single channels, it is difficult to choose among the possible mechanisms.

Voltage-Dependent Gating of Colicin Channels

The gating kinetics of the native colicin Ia channel are complex; changes in conductance occur on time scales from a fraction of a second up to hours. In addition to the four states shown in Fig. 7, there are probably states with the tip of the hydrophobic hairpin buried within the membrane, numerous stages in the voltage-dependent translocation of residues 474 to 541, and membrane-bound inactivated states. Many of these states can be eliminated by using *trans* streptavidin to anchor the hydrophobic segment in a transmembrane orientation, and then adding *cis* streptavidin to effectively remove the unanchored colicin. The resulting channel appears to have an open state similar to the wild-type channel (based on the single-channel conductance). It appears that this simplified version of colicin channel gating retains enough features of normal gating to be relevant.

We have begun to address the long-standing puzzle of the source of the voltage dependence of colicin channel gating. In the previously identified translocated region [20, 22], which moves across the membrane in response to voltage, there are 15 basic and 8 acidic residues, which are candidate voltage sensors. Additional gating charge could be contributed by the approximately 6 basic and 5 acidic residues in the flanking regions, which are presumed to form transmembrane segments in the open channel. Part of the problem in dissecting the contributions of the different residues has been the difficulty of measuring the voltage dependence at all. In an earlier study of the native colicin Ia, it was necessary to measure the conductance at a quasi-steady state, attained shortly after stepping the voltage, to avoid the subsequent slow increase in conductance attributable to the entry of new colicin into the membrane [16]. It may not be proper, however, to attribute this slow increase in conductance to new channels entering the membrane, given that the present study shows that channels already in the membrane also have a slow component of turn-on.

For the streptavidin-anchored colicin, we measured a gating charge of about 1.8, independent of pH. This is significantly less than the 3 to 6 gating charges (depending on pH) measured for the unanchored colicin [16] (or the 7 net positive charges translocated across the membrane [20]). This discrepancy presumably reflects an actual difference in the voltage dependence of anchored and native colicins. Given that there are no charged residues in the hydrophobic segment, we do not expect it to contribute directly to the gating charge. Nonetheless, its reorientation could be coupled to the translocation of charged residues elsewhere in the molecule. In that case, the transition between states 2 and 3 of our model could have some voltage dependence, perhaps accounting for part of the difference in gating charge measured for anchored and native colicins. Whether anchoring the hydrophobic segment across the membrane affects the translocation of other residues remains to be seen.

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Noted added in proof

A more complete version of reference 24 is in press: Elkins, P., Bunker, A., Cramer, W.A., Stauffacher, C.V. 1997. A mechanism for toxin insertion into membranes is suggested by the crystal structure of the channel- forming domain of calicin E1. *Structure* (*in press*)