

# Major Transmembrane Movement Associated with Colicin Ia Channel Gating

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**ABSTRACT** Colicin Ia, a bacterial protein toxin of 626 amino acid residues, forms voltage-dependent channels in planar lipid bilayer membranes. We have exploited the high affinity binding of streptavidin to biotin to map the topology of the channel-forming domain (roughly 175 residues of the COOH-terminal end) with respect to the membrane. That is, we have determined, for the channel's open and closed states, which parts of this domain are exposed to the aqueous solutions on either side of the membrane and which are inserted into the bilayer. This was done by biotinylating cysteine residues introduced by site-directed mutagenesis, and monitoring by electrophysiological methods the effect of streptavidin addition on channel behavior. We have identified a region of at least 68 residues that flips back and forth across the membrane in association with channel opening and closing. This identification was based on our observations that for mutants biotinylated in this region, streptavidin added to the *cis* (colicin-containing) compartment interfered with channel opening, and *trans* streptavidin interfered with channel closing. (If biotin was linked to the colicin by a disulfide bond, the effects of streptavidin on channel closing could be reversed by detaching the streptavidin–biotin complex from the colicin, using a water-soluble reducing agent. This showed that the cysteine sulfur, not just the biotin, is exposed to the *trans* solution.) The upstream and downstream segments flanking the translocated region move into and out of the bilayer during channel opening and closing, forming two transmembrane segments. Surprisingly, if any of several residues near the upstream end of the translocated region is held on the *cis* side by streptavidin, the colicin still forms voltage-dependent channels, indicating that a part of the protein that normally is fully translocated across the membrane can become the upstream transmembrane segment. Evidently, the identity of the upstream transmembrane segment is not crucial to channel formation, and several open channel structures can exist.

## INTRODUCTION

Colicin Ia, a plasmid-encoded, water soluble, single polypeptide of 626 amino acid residues (Mankovich et al., 1986), is a member of the class of colicins that form voltage-gated ion-conducting channels in bacterial plasma membranes and in artificial phospholipid bilayer membranes (Schein et al., 1978; for reviews see Slatin, 1988; Cramer et al., 1990; Lakey et al., 1994). Like all of the colicins for which domain analyses have been done, colicin Ia is assumed to have three domains: (1) the middle region, which binds to a receptor in the outer membrane of the target cells; (2) the NH<sub>2</sub>-terminal region, which, in conjunction with proteins in the target cell, transports the colicin to the plasma membrane; and (3) the COOH-terminal region (~175

amino acids), which is the channel-forming domain (for review see Cramer et al., 1995). The main issue addressed in this paper is the overall membrane topology of the channel-forming domain in the channel's open and closed states, i.e., which residues are exposed to the aqueous solutions on either side of the membrane and which are sequestered within the lipid bilayer. In the Discussion, we also comment upon several intriguing questions about channel structure and gating.

In a previous paper (Slatin et al., 1994), we reported the surprising finding that a stretch of at least 31 amino acid residues of colicin Ia, extending from residue 511 to 541, is translocated back and forth across the lipid bilayer in conjunction with channel opening and closing, whereas residues 544 and 547 are moved in and out of the bilayer but not entirely across it. Here we elaborate on this picture and report that, associated with channel gating, a stretch of 68 residues (474–541) is translocated entirely across the membrane, whereas some portion of the 29 residues (544–572) just down-

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stream is moved into and out of the bilayer; we postulate that this portion forms a transmembrane segment in the open state. Although we have evidence for another transmembrane segment just upstream of the translocated segment, its exact boundaries are not fixed and can be experimentally manipulated, resulting in structurally distinct open states for the colicin Ia channel.

## MATERIALS AND METHODS

### Mutagenesis and Biotinylation of Colicin Ia

Wild-type colicin Ia contains no cysteine residues. Unique cysteines were introduced by site-directed oligonucleotide mutagenesis, and the mutant proteins were expressed and purified as described previously (Jakes et al., 1990; Qiu et al., 1994). The purified proteins were biotinylated with one of the following sulfhydryl-specific reagents: 3-(*N*-maleimidylpropionyl) biocytin, *N*-(biotinoyl)-*N'*-(iodoacetyl)ethylenediamine (both from Molecular Probes, Inc., Eugene, OR), *N*-[6-(biotinamido)hexyl]-3'-(2'-pyridyldithio)propionamide, or *N*-[2-(biotinamido)ethyl]-3'-(2'-pyridyldithio)propionamide (both from Pierce Chemical Co., Rockford, IL). This last compound was synthesized for us by Dr. Paul Davis at Pierce; it is not currently listed in their catalog. The structures of these reagents and the resulting biotinylated products are shown in Fig. 1. Note that the product of the reactions with either of the last two reagents can be reduced to regenerate the original, unbiotinylated protein. Biotinylation with the first

two reagents was performed as described previously (Qiu et al., 1994; Slatin et al., 1994). For the latter two reagents, the protein was reduced with 20 mM dithiothreitol (DTT), transferred into degassed 0.2 M sodium borate, 2 mM EDTA, pH 9.0 buffer by means of an NAP-5 G-25 Sephadex column (Pharmacia LKB Biotechnology Inc., Piscataway, NJ), and 20 vol of the protein solution were incubated with 1 vol of the biotinylation reagent (4 mM in *N,N*-dimethylformamide) for 0.5–3 h at 31°C. The reaction mixture was then dialyzed at 4°C against 25 mM sodium borate, 2 mM EDTA, 300 mM NaCl, pH 9.0 (borate buffer). The extent of biotinylation was assessed by an SDS-polyacrylamide gel streptavidin-binding assay as described in Qiu et al., 1994. Generally, >80–90% of the mutant colicin was biotinylated. Nevertheless, to ensure that all of the channel activity observed on lipid bilayer membranes came from the biotinylated mutant, we usually purified the biotinylated protein on a column of monomeric avidin (Ultralink; Pierce Chemical Co.). Because the affinity for biotin of monomeric avidin is weaker than that of the usual tetrameric avidin or streptavidin, biotinylated proteins can be eluted from this column with biotin (Green and Toms, 1973). Columns were prepared and run exactly as specified in the instructions from Pierce. After the protein was loaded and washed extensively in PBS (10 mM sodium phosphate, 150 mM NaCl, pH 7.2) to remove any unbiotinylated proteins, the biotinylated protein was eluted from the column in 2 mM biotin-PBS. It was then concentrated by spinning in concentrators (Centricon 10; Amicon, Beverly, MA) and dialyzed extensively against borate buffer to remove biotin. Biotinylated colicin purified on monomeric avidin showed no evidence of any residual, unbiotinylated colicin in the gel assays. Minor impurities initially present in our original puri-

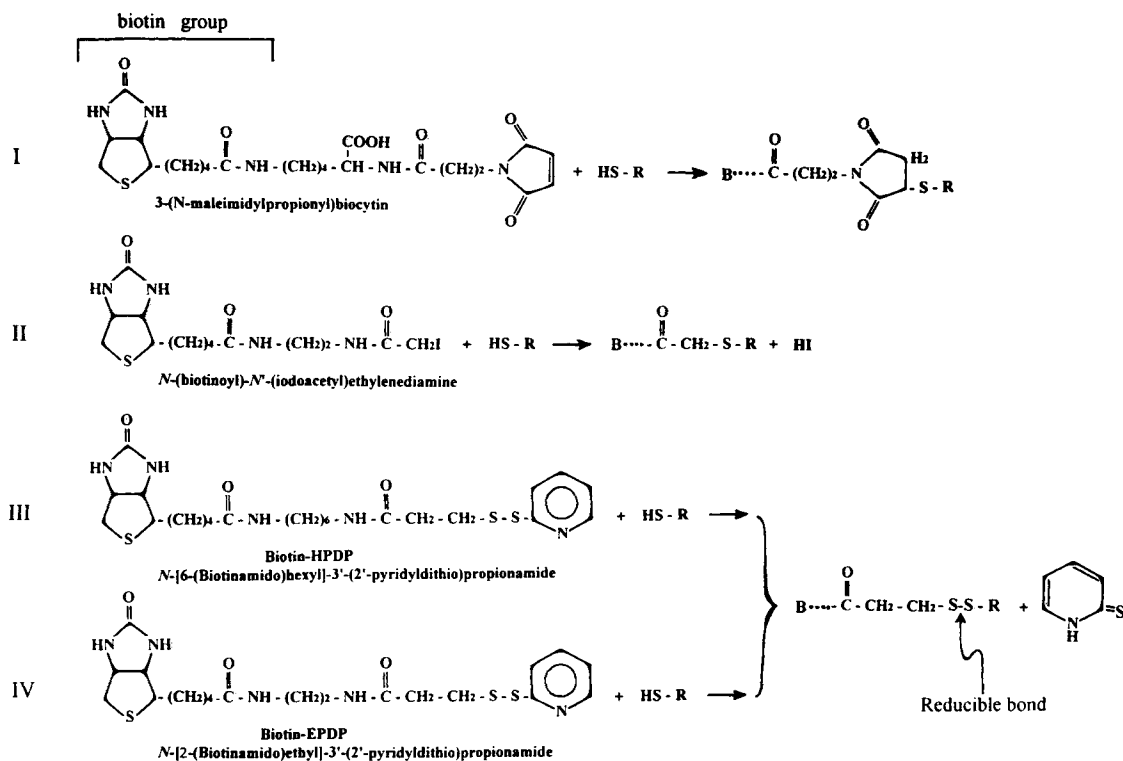


FIGURE 1. The biotinylation reagents used in these experiments and their reactions with the sulfhydryl group of cysteine. R—SH = mutant colicin with cysteine residue. For the products of the reactions, the biotin group and spacer arm are represented by B···. Note that for reagents III and IV, the disulfide bond of the products can be reduced to regenerate the original unbiotinylated colicin.

fied colicin were also removed by the avidin column. Streptavidin was obtained from either Calbiochem Corp., La Jolla, CA, or Pierce Chemical Co.

### Planar Bilayer Experiments

Membranes were formed at room temperature from asolectin (lecithin type IIS; Sigma Chemical Co., St. Louis, MO) from which nonpolar lipids had been removed (Kagawa and Racker, 1971). Lipid (1% in pentane) was layered on top of the aqueous solutions in two compartments on either side of a Teflon partition. The partition contained an 80–120- $\mu\text{m}$  hole which was pre-treated with 3% squalene in petroleum ether. After the solvents evaporated, the lipid layers were raised over the level of the hole, forming the membrane (Montal, 1974). The volume of solution on each side of the membrane was  $\sim 1$  ml and contained 100 mM KCl, 5 mM  $\text{CaCl}_2$ , 1 mM EDTA, and an appropriate buffer; unless otherwise indicated, the buffer was either 10–50 mM MES, pH 6.2, or 20–50 mM HEPES, pH 8.0. The transmembrane voltage was clamped, and the resulting current was monitored as previously described (Jakes et al., 1990); 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 0.5–7 mg/ml), either biotinylated or not, was usually diluted 1:1 (vol:vol) with 1% octylglucoside (Calbiochem) before the addition of  $\sim 1$   $\mu\text{l}$  to the *cis* compartment; dilution with octylglucoside yielded more channels per milligram of protein without affecting channel behavior. In single-channel experiments, the KCl concentration was 1 M, and octylglucoside was frequently omitted. Stock solutions of wild-type colicin Ia and cysteine mutants were stored frozen at  $-70^\circ\text{C}$  in 300 mM NaCl, 25 or 50 mM sodium borate, 2 mM EDTA, pH 9.0, at concentrations  $>1$  mg/ml; thawed aliquots and biotinylated mutants were kept at  $4^\circ\text{C}$ , where they generally retained channel-forming activity for months.

### Experimental Rationale

Colicin Ia induces a characteristic voltage-dependent response in planar lipid bilayer membranes: current increases in response to *cis*-positive voltages and rapidly decreases to zero at negative voltages (Nogueira and Varanda, 1988). This macroscopic response, which is the one primarily assayed in our experiments, reflects the opening and closing of many individual channels. By using site-directed mutagenesis to introduce a cysteine, followed by cysteine-specific biotinylation with one of the reagents shown in Fig. 1, one can attach a biotin at a specific site in colicin Ia. This site then serves as a target for the binding of streptavidin, a 60-kD water-soluble protein that binds nearly irreversibly to biotin (Wilchek and Bayer, 1988; Hashimoto et al., 1986; Chaiet and Wolf, 1964). Binding of streptavidin from one side of the membrane or the other might be expected to interfere with voltage gating, if the biotinylated site moves during the gating process. In this way, one may hope to determine whether a given residue is accessible to streptavidin in the *cis* solution or the *trans* solution, or resides in the interior of the bilayer (and is therefore inaccessible to streptavidin) when the channels are open or closed.

From preliminary data and by analogy to the other channel-

forming colicins, the channel-forming region of colicin Ia is presumed to reside in the last 175 amino acid residues, extending roughly from residue 451 to 626 (Cramer et al., 1995) (see Fig. 2). Near the carboxy terminus is a hydrophobic segment consisting of 40 uncharged amino acids (residues 573–612); this segment is believed to be crucial for interaction with the membrane and is assumed to remain buried in the membrane interior during channel gating (Cleveland et al., 1983). All of the other channel-forming colicins have a similar segment, ranging in length from 35 to 49 amino acids. We comment on this hydrophobic segment in the Discussion, but it is the region upstream from this (from residues 454 to 572) that is the subject of this work. Results obtained for some of the residues in this region have been reported previously (Qiu et al., 1994; Slatin et al., 1994).

## RESULTS

Before presenting our specific findings, we note the following. First, biotinylation did not qualitatively change the voltage gating of the channels, although at some sites (e.g., 544 [see Qiu et al., 1994]), it did alter the kinetics. Thus, we can assume that the basic gating mechanism is unaffected by biotinylation. Second, the effect of streptavidin on a given biotinylated mutant was not dependent on which of the four biotinylating reagents (Fig. 1) was used. We initially used reagent I because of our familiarity with its chemistry, and duplicated our results at certain sites with reagent II, which attaches the biotin by a shorter spacer arm (Slatin et al., 1994). Most of our subsequent experiments were done with reagent III, which has the advantage that its product is reducible (particularly at basic pH) by DTT or cysteine, and thereby regenerates the original, unbiotinylated protein (see Fig. 1). Most recently, we have used reagent IV, which was synthesized for us; it combines the reversibility of reagent III with the short spacer arm of reagent II. We comment on the issues of spacer arm length and reversibility later.

We used the following protocol in our experiments:

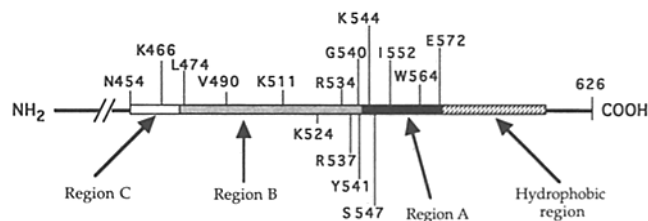
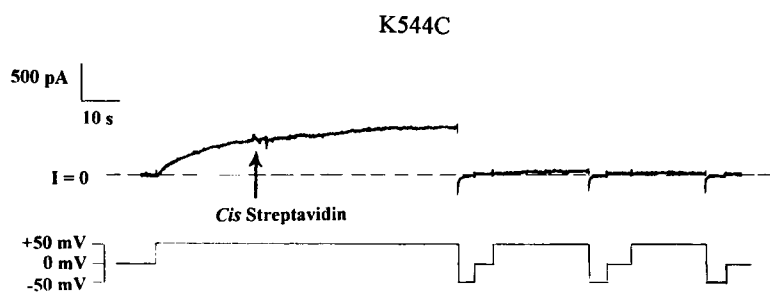


FIGURE 2. Linear diagram of the channel-forming region (residues 451–626) of colicin Ia, indicating the residues that we mutated to cysteine and then biotinylated. Also designated in the figure are four regions: the hydrophobic region, which we assume is inserted into the bilayer in both the closed and open states of the channel; regions A and C, parts of which move into and out of the bilayer when the channel opens and closes; and region B, which moves all the way across the bilayer, back and forth, when the channel opens and closes.



this point the voltage was pulsed to  $-50$  mV and the current rapidly decayed to zero. The subsequent  $+50$  mV pulse induced a barely perceptible current, and the one after that induced no measurable current, indicating complete killing of channel activity by *cis* streptavidin. Solutions on both sides of the membrane were 100 mM KCl, 5 mM  $\text{CaCl}_2$ , 1 mM EDTA, 50 mM MES, pH 6.2. The biotinylation of K544C was with reagent I in Fig. 1.

(a) we determined that the conductance induced by a particular biotinylated mutant behaved normally; that is, it turned on at positive voltages and off at negative voltages (typically  $\pm 50$  mV); (b) we then added streptavidin to either the *cis* or *trans* solution to a final concentration of 10–40  $\mu\text{g}/\text{ml}$ ; and (c) we monitored the subsequent responses to positive and negative voltages. To facilitate the following discussion, we have divided the sequence of colicin Ia between residues 454 and 572 into three contiguous regions, which we have creatively designated A, B, and C, as shown in Fig. 2. As will be seen, biotinylated residues in region B are sensitive to *trans* streptavidin, whereas those in regions A and C are not.

*Residues 544–572 (Mutants K544C, S547C, I552C, W564C, and E572C): Region A*

*Effect of trans streptavidin.* We could detect no effect of *trans* streptavidin on the voltage gating of channels formed by the biotinylated mutants in region A (data not shown).

*Effect of cis streptavidin.* When added to the *cis* side of the membrane, streptavidin drastically reduced, essentially to zero, the conductance induced by biotinylated mutants in region A (shown for K544C in Fig. 3). Addition of free biotin after the *cis* streptavidin effect did not restore channel activity, which is consistent with the very tight binding of streptavidin to biotin (results not shown). Streptavidin had no effect on channels formed from unbiotinylated mutants, nor from biotinylated mutants if sufficient free biotin was added to the *cis* solution before streptavidin addition. Thus the *cis* streptavidin effect resulted from specific streptavidin binding to the biotin moiety on the colicin.

*Cis* streptavidin prevented closed biotinylated channels from opening, but it did not destroy the activity of channels that were already open. The latter point was apparent from experiments in which streptavidin was added to the *cis* solution while the channels were held

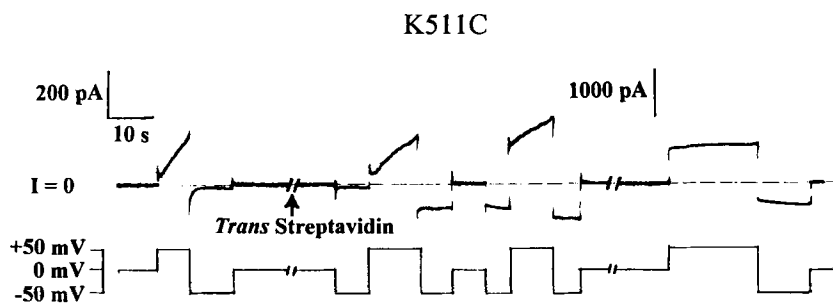
open with a large positive voltage ( $\geq +50$  mV). Under these circumstances, the conductance persisted for many minutes. After the channels were closed by a negative voltage pulse, however, the conductance did not return in response to the same positive potential (e.g.,  $+50$  mV), presumably because the closed channels had now bound to streptavidin.

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*Residues 474–541 (Mutants L474C, V490C, K511C, K524C, R534C, R537C, G540C, and Y541C): Region B*

*Effect of trans streptavidin.* Typically, the gating behavior seen after addition of streptavidin to the *trans* solution was that shown in Fig. 4 for the K511C biotinylated mutant. After each successive opening of the channels at  $+50$  mV, a greater fraction failed to turn off at  $-50$  mV, until eventually there was no further turn-on and turn-off of channels at  $\pm 50$  mV, because all of them had been locked into an open state.<sup>1</sup> Thus, when the channels were open, the biotinylated residue was exposed to streptavidin in the *trans* solution, which bound to the biotin moiety and prevented the channels from closing. This effect of *trans* streptavidin was not seen with channels formed from unbiotinylated mutants, nor with wild-type colicin Ia (which contains no cysteine) that had been taken through the biotinylation procedure. Neither was it seen if sufficient free biotin was added to the *trans* compartment before streptavidin addition. Thus the *trans* streptavidin effect resulted from

<sup>1</sup>In some *trans* streptavidin experiments, including the one shown in Fig. 4, the conductance could be seen to increase slightly in response to sustained  $-50$  mV pulses, rather than remain strictly constant. This no doubt reflects the complexity of colicin gating, which has been noted often (Slatin et al., 1986; Slatin, 1988; Collarini et al., 1987; Abrams et al., 1991; Bullock, 1992; Cássia-Moura, 1993). The binding of *trans* streptavidin obviously prevents the channels from closing, but it may well permit some traffic among open states, or perhaps between open states and “inactivated” states (Slatin et al., 1986). In this paper, we consider only an admittedly oversimplified gating scheme, leaving elucidation of the fine structure for future research.



was not held long enough at +50 mV for the current to start leveling off, so the response appears as an almost linear rise in current.) During the first break in the record (30 s), 40  $\mu\text{g}$  of streptavidin were added to the *trans* compartment. Subsequently, there was not complete turn-off of current at -50 mV, after a +50-mV stimulation of current, and the magnitude of the current at -50 mV increased as more turn-on was induced by +50-mV stimuli. Eventually (the second break in the record was 4.5 min), there was no additional current induced by a +50-mV stimulus; all of the channels were locked into an open state by *trans* streptavidin (note the change of scale). Notice, however, that the response to a  $\pm 50$ -mV stimulus was not ohmic; the current at +50 mV was 1.6 times larger than that at -50 mV. Also note that the current in the presence of *trans* streptavidin was not noticeably noisier than that before streptavidin addition; this should be contrasted to the record in Fig. 5, showing the *trans* streptavidin effect on the current induced by biotinylated Y541C. Also compare the speed of the development of the response to *trans* streptavidin in this record for biotinylated K511C with that in Fig. 5 for biotinylated Y541C. Solutions on both sides of the membrane were as in Fig. 3. The biotinylation of K511C was with reagent I in Fig. 1.

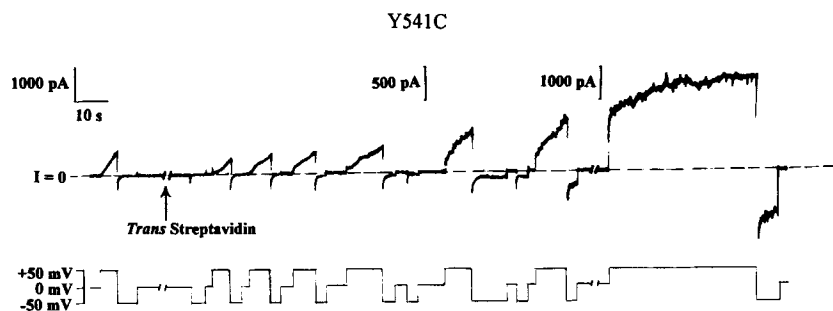
specific streptavidin binding to the biotin moiety on the protein. The effect of streptavidin was not reversed by the subsequent addition of excess biotin to the *trans* solution.

The details of the response to *trans* streptavidin were mutant dependent. With some biotinylated mutants, the current at positive voltages became much noisier after streptavidin binding than before its interaction (Fig. 5), whereas with others (as in Fig. 4), there was little or no excess noise. In addition, the "locked-in" conductance of all the mutants at -50 mV was less than that at +50 mV, perhaps resulting from interaction of bound streptavidin with the open channel<sup>2</sup> (Fig. 4). For

<sup>2</sup>This rectification was greater than that observed for single channels in the absence of streptavidin. Ordinarily, the conductance of single colicin Ia channels is about 30% greater at +50 mV than at -50 mV.

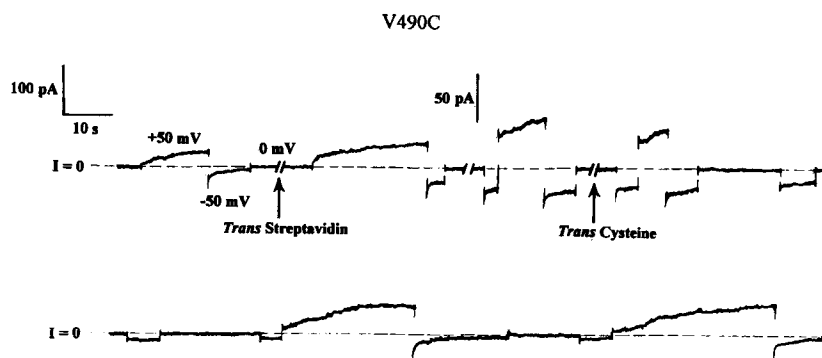
some mutants, particularly biotinylated G540C and Y541C, the response to *trans* streptavidin developed very slowly over many minutes, as if the biotin moiety was not easily accessible to *trans* streptavidin. With other mutants, such as biotinylated V490C and K511C, the response developed within a few seconds.

*Reversal of the trans streptavidin effect with reducing agents.* Since biotin is attached to a cysteine residue on the colicin by a spacer arm (see Fig. 1), it is possible that, although the attached biotin is exposed to the *trans* solution, the cysteine residue itself could be buried in the bilayer as much as 16 Å (reagent I) or 8 Å (reagent II) from the membrane-solution interface. We addressed this issue by using reagents III and IV to biotinylate the residues. If the disulfide linkage to the cysteine residue can be broken with a water-soluble reducing agent, thereby regenerating the original unbi-



was not held long enough at +50 mV for the current to start leveling off, so the response appears as an almost linear rise in current.) During the first break (1 min), 10  $\mu\text{g}$  of streptavidin were added to the *trans* compartment. Very slowly over time, there developed a residual current that did not turn off at -50 mV. (Compare the kinetics of the development of this response to *trans* streptavidin of biotinylated Y541C to that of biotinylated K511C in Fig. 4.) The magnitude of this current at -50 mV increased as more turn-on was induced by +50-mV stimuli. Note that the current in the presence of *trans* streptavidin eventually becomes much noisier than that before streptavidin addition. The second break in the record was 4.5 min. Solutions on both sides of the membrane were as for Fig. 3. The biotinylation of Y541C was with reagent I in Fig. 1.

FIGURE 5. The effect of *trans* streptavidin on the conductance induced by a biotinylated mutant in Region B: an example with excess noise. Several minutes before the start of the record, 0.9  $\mu\text{g}$  of biotinylated Y541C (without octylglucoside) was added to the *cis* compartment, and the membrane was pulsed several times between  $\pm 50$  mV to confirm normal voltage gating. The record begins with one such  $\pm 50$ -mV sequence, and we see normal turn-off of current at -50 mV. (The voltage



break in the record (90 s), 40  $\mu$ g of streptavidin were added to the *trans* compartment. Subsequently, there was not complete turn-off of current at  $-50$  mV, and the magnitude of this current at  $-50$  mV increased as more turn-on was induced by  $+50$ -mV stimuli. During the last break in the record (60 s), cysteine was added to the *trans* compartment to a concentration of 40 mM. Over time (continuing on the second line of the figure), the current at  $-50$  mV declined, and finally the normal gating at  $\pm 50$  mV was restored. Thus, cysteine had cleaved off the biotin, with its attached streptavidin, from biotinylated V490C, regenerating the original unbiotinylated V490C. The solutions on both sides of the membrane were 100 mM KCl, 5 mM CaCl<sub>2</sub>, 1 mM EDTA, 20 mM HEPES, pH 7.5. The biotinylation of V490C was with reagent III of Fig. 1. The middle break in the record was 30 s.

otinylation mutant, we could conclude that the sulfur of the cysteine residue is exposed to the *trans* solution and not buried within the bilayer.

We used this strategy with the biotinylated mutants L474C, V490C, K511C, K524C, R534C, and R537C. These experiments were generally done at pH 8.0, because the reduction reaction is faster at basic pH. The *trans* streptavidin effect was reversed by reducing agents such as cysteine and DTT.<sup>3</sup> An example of this reversal is shown in Fig. 6: after the channels were locked into the open state by *trans* streptavidin, addition of cysteine to the *trans* solution restored normal channel gating (i.e., channels turned off at  $-50$  mV and turned on at  $+50$  mV). In all experiments, addition of cysteine to the *trans* solution, but not to the *cis* solution, reversed the *trans* streptavidin effect, and thereby confirmed the *trans* side location of the disulfide bond linking biotin to the colicin. In contrast, DTT added to either the *cis* or *trans* solution reversed the *trans* streptavidin effect. Apparently, DTT is sufficiently permeant through the bilayer that it attains an effective concentration at the *trans* interface even when added to the *cis* solution.

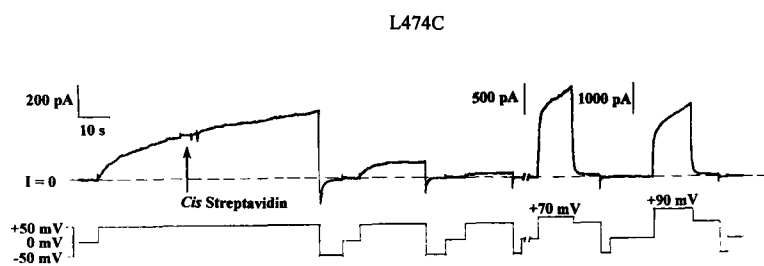
**Effect of *cis* streptavidin.** The impression obtained from our earlier *cis* streptavidin experiments (Slatin et al., 1994) was that colicin Ia channels cannot open when streptavidin is bound to a residue in region B. Although *cis* streptavidin did not completely kill channel-

forming activity, i.e., there was some residual activity, particularly at voltages greater than  $+50$  mV, we had previously attributed this to the small contamination (on the order of a few percent) of the biotinylated mutant colicin by unbiotinylated mutant. We show now, however, that even after unbiotinylated colicin was removed on a monomeric avidin column (see Materials and Methods), thus eliminating this source of residual activity, *cis* streptavidin still did not block 100% of the channel-forming activity of region B mutants, but instead altered their voltage gating.

A dramatic example of the nature of the activity that can arise in the presence of *cis* streptavidin occurred with biotinylated L474C (Fig. 7). At  $+50$  mV, *cis* streptavidin virtually eliminated channel activity. If this were the only voltage tested, one would conclude that channels cannot form with streptavidin bound to residue 474 on the *cis* side. At  $+70$  mV and higher, however, even larger conductances could be achieved than existed before streptavidin addition.<sup>4</sup> Moreover, the steep section of the conductance-voltage curve was extended to higher voltages, so that there was substantial voltage-dependent gating between  $+70$  and  $+90$  mV, whereas there was little additional conductance induced by voltages above  $+50$  mV in the absence of *cis* streptavidin. The conductance that developed in the presence of *cis* streptavidin, in contrast to that in its absence, was not, of course, affected by *trans* streptavidin (data not shown). Biotinylated L474C preincubated with streptavidin in-

<sup>3</sup>The *cis* streptavidin effect was also reversed by these reducing agents, when added to the appropriate side, not only for channels formed by mutants biotinylated in region B, but also for those biotinylated in regions A and C. This, however, is not a particularly informative finding, since reduction on the *cis* side removes biotin from a large pool of closed channels, which is functionally equivalent to adding unbiotinylated colicin to the *cis* compartment.

<sup>4</sup>This description applies to experiments at pH 8.0. A similar description applies to experiments at pH 6.2, except at 20 mV smaller voltages. That is, at pH 6.2, *cis* streptavidin virtually eliminated channel activity at  $+40$  mV, but at  $+50$  mV and higher, even greater conductances could be achieved than existed prior to streptavidin addition.



to rise. After 50 s, the voltage was pulsed to  $-50$  mV, and the current rapidly decayed to zero. A subsequent  $+50$ -mV pulse induced a much smaller current than that before streptavidin addition, and a second  $+50$ -mV pulse induced almost no current response. Thus, it appeared that the biotinylated L474C-induced conductance had been almost completely killed by *cis* streptavidin. However, subsequent  $+70$ - and  $+90$ -mV pulses induced an even larger conductance than existed before the addition of *cis* streptavidin (note the changes in scale). Note also the rapid decay of the current to almost zero when the voltage was pulsed from  $+70$  or  $+90$  mV to  $+50$  mV. The solutions on both sides of the membrane were 100 mM KCl, 5 mM CaCl<sub>2</sub>, 1 mM EDTA, 50 mM HEPES, pH 8.0. The biotinylation of L474C was with reagent III of Fig. 1. The break in the record was  $\sim 1$  min.

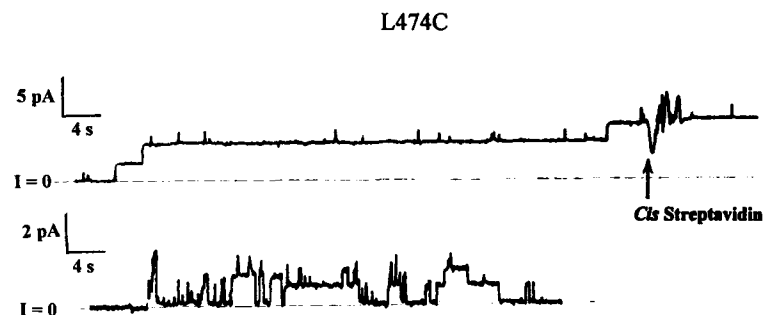
duced the same altered voltage-dependent gating as that (described above) resulting from the addition of streptavidin to the *cis* solution; these channels were also unaffected by *trans* streptavidin. Given that residue 474 is “normally” on the *trans* side in the channel’s open state (see previous section), this result identifies two distinct open states: one with residue 474 on the *cis* side of the membrane and one with residue 474 on the *trans* side. These states are distinguished both by differences in voltage dependence, as described above, and also by differences in single-channel conductance and lifetimes. As seen in Fig. 8, when residue 474 was bound by *cis* streptavidin, the single channel conductance was less than half that of unbound channels. Furthermore, at voltages where unbound channels were essentially always open, the bound channels flickered between open and closed states on a time scale of seconds.

Similar, though less pronounced, behavior was seen with biotinylated V490C in the presence of *cis* streptavidin. Again, the dampening effect of *cis* streptavidin on channel formation could be overcome at large positive voltages, but for this mutant even larger voltages were

required, and the conductance turn-on was faster and noisier than in the absence of streptavidin (Fig. 9 a). This trend continued for biotinylated K511C (Fig. 9 b) and R534C (Fig. 9 c). For the latter, voltage-dependent conductance developed only for voltages  $\geq +80$  mV, and even at  $+110$  mV, the conductance was only a fraction of its value before the addition of *cis* streptavidin. In summary, the voltage-dependent conductance in the presence of *cis* streptavidin is nearest to normal with biotinylated L474C and becomes progressively less so (in terms of kinetics, voltage range, and noise) the more COOH-terminal the residue. In no case was there an effect of *trans* streptavidin on conductance after the binding of *cis* streptavidin to the colicin.

#### Interpretation of the *cis* and *trans* Streptavidin Experiments Thus Far Presented for Residues 474–572 (Regions A and B of Fig. 2)

We present here our interpretation of the preceding findings, as it makes the motivation and analysis of the subsequent experiments more comprehensible. All of



the channels had been turned off by a  $-50$ -mV pulse, enabling them to react with *cis* streptavidin. (This particular trace happens to have been taken  $\sim 15$  min after the addition of streptavidin.) A mixture of channel sizes can be seen, most of which are of much smaller conductances than that obtained before the addition of streptavidin. (Note the change in current scale.) Also note that these channels flicker on and off at  $+50$  mV, in contrast to the behavior before the addition of streptavidin to the *cis* compartment. The solutions on both sides of the membrane were 1 M KCl, 5 mM CaCl<sub>2</sub>, 1 mM EDTA, 20 mM MES, pH 6.2. The biotinylation of L474C was with reagent III in Fig. 1.

FIGURE 8. The effect of *cis* streptavidin on biotinylated L474C at the single-channel level. Several minutes before the beginning of the record, 27 ng of biotinylated L474C were added to the *cis* compartment. Both the upper and lower traces show the current response in the presence of a maintained  $+50$ -mV stimulus. In the upper trace there are three channel openings, each of  $\sim 2.5$  pA. Note that throughout the course of this trace, the channels did not close. At the arrow,  $10 \mu\text{g}$  of streptavidin were added to the *cis* compartment. The lower trace is an example of the record obtained after streptavidin addition and after

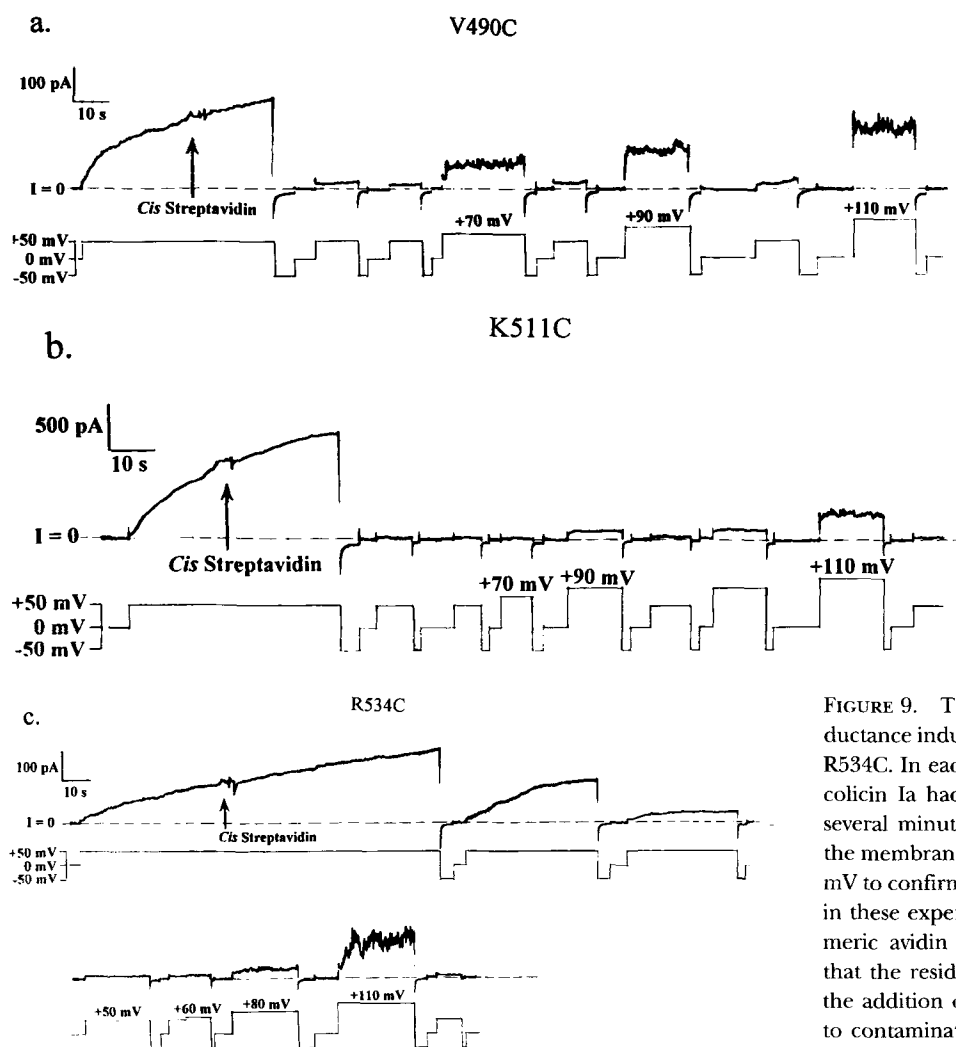


FIGURE 9. The effect of *cis* streptavidin on the conductance induced by biotinylated V490C, K511C, and R534C. In each experiment, the biotinylated mutant colicin Ia had been added to the *cis* compartment several minutes before the start of the record, and the membrane was pulsed several times between  $\pm 50$  mV to confirm normal gating. Also, the samples used in these experiments had been purified on a monomeric avidin column (Materials and Methods), so that the residual activity seen in these records after the addition of *cis* streptavidin cannot be attributed to contamination by unbiotinylated protein. (a) Biotinylated (with reagent III of Fig. 1) V490C. There

were 1.4  $\mu\text{g}$  of colicin plus 5  $\mu\text{g}$  octylglucoside in the *cis* solution. At the arrow, 40  $\mu\text{g}$  of streptavidin were added to the *cis* compartment. Note that the subsequent +50-mV pulses induced very little current, but there were very noisy currents with a rapid rise time induced by +70, +90, and +110 mV stimuli. (b) Biotinylated (with reagent II in Fig. 1) K511C. 1  $\mu\text{g}$  of colicin plus 15  $\mu\text{g}$  of octylglucoside were in the *cis* solution. At the arrow, 40  $\mu\text{g}$  of streptavidin were added to the *cis* compartment. Note that the subsequent +50- and +70-mV pulses induced essentially no current, but there were small currents with a rapid rise time induced by +90- and +110-mV stimuli. (c) Biotinylated (with reagent III in Fig. 1) R534C. There were 0.7  $\mu\text{g}$  of colicin plus 10  $\mu\text{g}$  of octylglucoside in the *cis* solution. At the arrow, 10  $\mu\text{g}$  of streptavidin were added to the *cis* compartment. Eventually (trace continued on the second line), there was almost no current induced by a +50- or +60-mV stimulus. There were, however, very noisy currents with a rapid rise time induced by +80- and +110-mV stimuli. (The slower time course for the *cis* streptavidin effect to develop in this record, as compared to *a* and *b*, could be due to slower mixing in of the streptavidin, the smaller (one-fourth) amount of streptavidin used, intrinsically poorer access of streptavidin to biotin on residue 534, or any combination of these factors.) The solutions on both sides of the membrane for all records were 100 mM KCl, 5 mM  $\text{CaCl}_2$ , 1 mM EDTA, 20 mM MES, pH 6.2.

the residues from 474 to 572, and presumably everything upstream, reside on the *cis* side of the membrane when the channel is closed. In the "normal" open state, the residues in region B (474–541) are accessible to *trans* streptavidin (we have noted earlier that channels can still open when residues within this region are held on the *cis* side by *cis* streptavidin), whereas the residues in region A (544–572) are not. Thus, associated with the opening of a channel, region B is translocated

across the membrane from the *cis* to the *trans* side, whereas part or all of region A is moved from the *cis* side to the bilayer interior (Fig. 10). We note, however, that region A is very long for a single membrane-spanning segment.

The biological analogue of the *cis* side of the membrane is the outside of the target bacterium, or the periplasmic face of its inner membrane. The amino-terminal two-thirds of the colicin includes its outer mem-



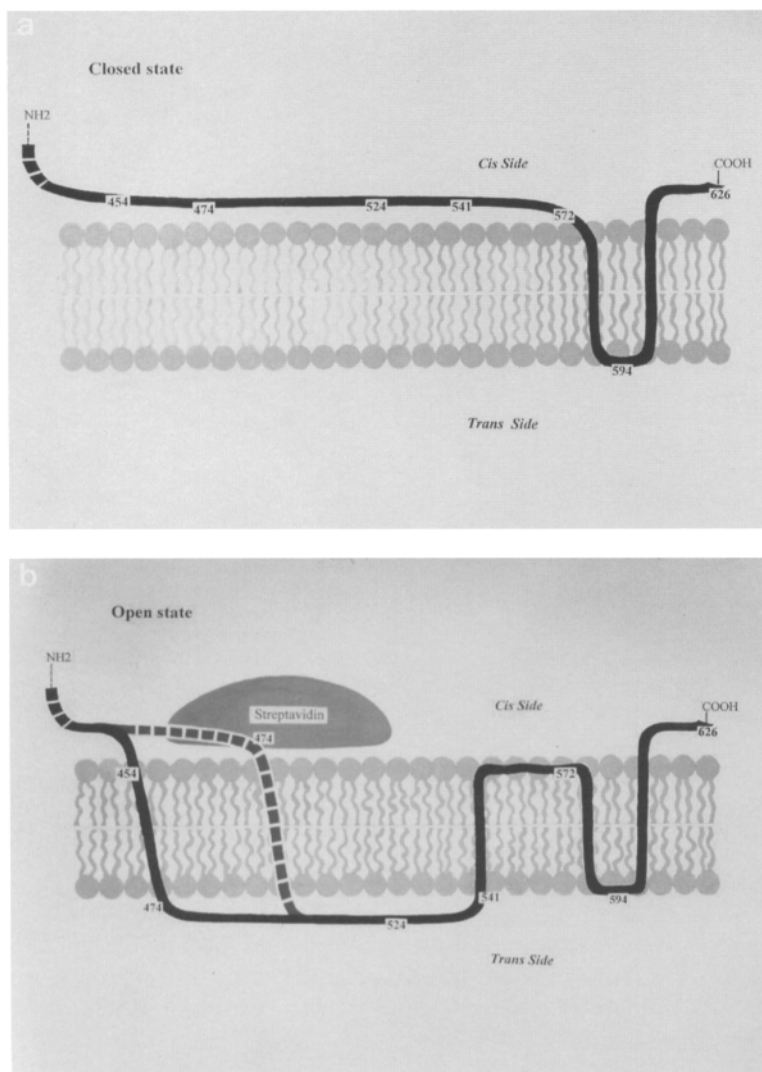


FIGURE 10. Model of the membrane topology of the colicin Ia molecule in the closed and open states of the channel. The hydrophobic segment is modeled as an  $\alpha$ -helical hairpin, with its tip near residue 594, which traverses the membrane in both the open and closed states, and is presumed to help anchor the colicin to the bilayer. (The analogous hydrophobic segment of colicin A is an  $\alpha$ -helical hairpin in the crystal structure [Parker et al., 1989].) In the closed state, all of the residues upstream from this are on the *cis* side of the membrane. In the open state, a segment between residues 541 and 572 and a segment from slightly before residue 454 to near residue 474 are inserted into the bilayer. The entire region between residues 474 and 541 inclusive is on the *trans* side of the membrane. Thus, the opening and closing of the channel is accompanied by the translocation of at least 68 residues (474–541) back and forth across the bilayer. The dashed portion shows that when residue 474 is held on the *cis* side by streptavidin, an alternative region becomes the upstream bilayer-inserted segment.

brane receptor-binding domain as well as the domain responsible for its transfer from the outer to the inner bacterial membrane. These domains probably remain outside the cytoplasm of the target cell (Bénédicti et al., 1992; Duché et al., 1995), and we assume that they likewise remain on the *cis* side of the bilayer in our system. For that arrangement to exist at the same time that residue 474 is accessible to *trans* streptavidin in the open channel state, there must be a transmembrane segment upstream of residue 474. Since the channel-forming region of colicin Ia apparently begins around residue 451 (Ghosh et al., 1993), this transmembrane segment should lie between residues 451 and 474. If this segment (region C) behaves like region A (residues 544–572), then mutants biotinylated in region C should behave analogously: namely, *cis* streptavidin should bind to closed channels and prevent them from opening, and open channels should be unaffected by both *cis* and *trans* streptavidin. However, given that

channels can open with residue 474 held on the *cis* side by streptavidin, it is possible that channels can also open with residues in region C held on the *cis* side. The following section describes our actual findings for this region.

#### *Residues 454–466: Region C (Fig. 2)*

*Effect of trans streptavidin.* The effect of *trans* streptavidin on biotinylated N454C and K466C channels was problematic and inconsistent. In most experiments, either channel gating was unaffected, or a small fraction of the conductance failed to turn off at negative voltages. In several experiments with biotinylated K466C, however, there was a significant suppression of channel turn-off, similar to that seen in region B (474–541), and even a turn-on of conductance at negative voltages, which could become greater than the conductance at positive voltages (data not shown). Our general impres-

sion is that the location of residues 454 and 466 in the open channel state is not fixed, and that this region may meander from within the bilayer to the *trans* side, the relative time spent in these locations being a function of undefined parameters. The flexibility of the location of the upstream region of the channel-forming domain is considered further in the Discussion.

**Effect of *cis* streptavidin.** When added to the *cis* side of the membrane, streptavidin altered the voltage dependence of channels formed by the biotinylated mutants N454C and K466C, thereby demonstrating that residues 454 and 466 are exposed to the *cis* solution. The effect was not the elimination of channel activity seen for channels biotinylated in region A (544–572) (Fig. 3); it was more like that described earlier for biotinylated L474C channels (Fig. 7). *Cis* streptavidin bound only to closed channels; it did not bind to open ones. This was evident in experiments in which streptavidin was added to the *cis* solution while the channels were held open with a large positive voltage ( $\geq 50$  mV). In Fig. 11, for example, the first time that the voltage was stepped down from +50 to +40 mV (when only open channels had been exposed to *cis* streptavidin), the current relaxed to a level only slightly below the “ohmic” 20% drop in driving force. (Typically, unmodified colicin Ia channels at this upper end of the range of their voltage dependence show little or no relaxation.) Later in the record, the channels were closed by a pulse to –50 mV (thus exposing the biotinylated residue to *cis* streptavidin in the closed state), and then reopened with a +50 mV pulse. Subsequently, the current displayed an altered voltage dependence: when the voltage was stepped down to +40 mV, the current relaxed to less than half its value at +50 mV. (Note also the

slightly increased noise at +50 mV compared to earlier in the record.) Other experiments with biotinylated N454C and K466C followed the same protocol, except that excess biotin was added to the *cis* solution after streptavidin addition, but before stepping to +40 mV. In this case, there was no current relaxation after the channels had been turned off at –50 mV, since there was no active streptavidin available to interact with the closed channels. Such results demonstrate that *cis* streptavidin binds to biotinylated N454C and K466C only in the closed state.

#### *Outside the Channel-forming Domain: Residue 402*

To serve as a control for mutant colicins biotinylated in the channel-forming domain, we biotinylated S402C, a residue thought to lie outside the channel-forming domain. We detected no effect of *trans* streptavidin on the voltage gating of this biotinylated mutant. *Cis* streptavidin had no effect on voltage gating either, although it caused the channel current noise to increase slightly (data not shown). Furthermore, biotinylated S402C prebound to streptavidin before its addition to the chamber displayed normal voltage gating and slightly increased current noise, similar to that induced by *cis* streptavidin. These results demonstrate that residue 402 is exposed to the *cis* solution, and that channels gate normally with *cis* streptavidin bound at this residue. This is consistent with the assignment of residue 402 to a location outside of the channel-forming domain, and supports our assumption that region C (or, in any case, some residues between 402 and 474) forms a transmembrane segment in the open channel. The small increase in noise could be due to interaction of

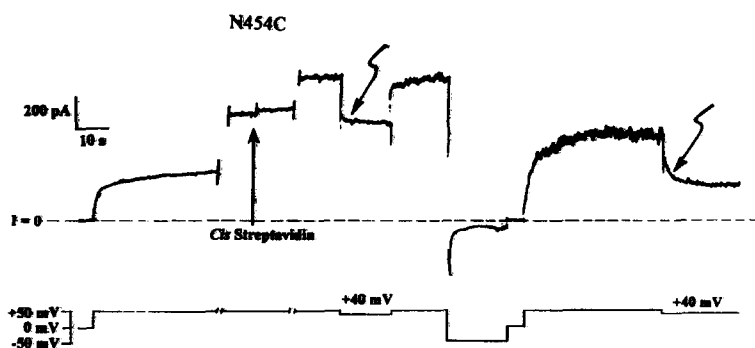


FIGURE 11. Demonstration that residue 454 is not exposed to the *cis* solution when the channels are open, but is exposed when the channels are closed. Several minutes before the start of the record, 1.5  $\mu$ g of biotinylated N454C (plus 15  $\mu$ g of octylglucoside) were added to the *cis* compartment, and the membrane was pulsed several times between  $\pm 50$  mV to confirm normal gating. In the record, during a prolonged stimulus of +50 mV (first break in the record was 3 min) with the colicin-induced current rising, 20  $\mu$ g of streptavidin were added to the *cis* compartment. After the current reached a steady level (second break in the record was 4 min), the voltage was

stepped down from +50 to +40 mV. The current relaxed to a level only slightly below the ohmic 20% drop in driving force (*first crooked arrow*). (The small relaxation was probably due to channels that opened after streptavidin addition.) After the channels were closed by a –50-mV stimulus and then reopened by a +50-mV stimulus, the voltage was again stepped down from +50 to +40 mV. This time, the current relaxed to a level less than half its value at +50 mV (*second crooked arrow*). This alteration in voltage dependence shows that biotinylated residue N454C was not bound by *cis* streptavidin while the channels were in the open state, but was bound when the channels were in the closed state. Solutions on both sides of the membrane were 100 mM KCl, 5 mM CaCl<sub>2</sub>, 1 mM EDTA, 20 mM MES, pH 6.2. The biotinylation of N454C was with reagent III in Fig. 1, and the sample was purified on the monomeric avidin column, as described in Materials and Methods.

the large streptavidin molecule with the channel, even if it is bound to a residue some distance from the channel.

*Do Residues in Region B (474–541) Slide Back and Forth across the Membrane in the Open Channel State?*

Our interpretation of the *trans* streptavidin experiments is that residues 474–541 reside on the *trans* side of the membrane when the colicin Ia channels are open (Fig. 10). Another possible interpretation is that the peptide chain in region B snakes back and forth across the membrane over time; a given biotinylated residue might be “caught” by *trans* streptavidin as that residue appears transiently on the *trans* side, thereby locking that particular channel in the open state. In other words, all of these residues might not be on the *trans* side simultaneously. This possibility is also consistent with the results described earlier; that is, channels could still open even if a residue normally accessible to *trans* streptavidin in the open state was first bound to *cis* streptavidin in the closed state. It is also suggested by the flexibility of region C (454–466) implied by the results described in an earlier section.

If any part of region B snakes back to the *cis* side while the channels are open, it would be susceptible to binding by *cis* streptavidin during prolonged openings. To test this, the type of experiment shown in Fig. 12 *a* was carried out for several biotinylated mutants in region B. After the conductance had risen to a more-or-less steady value at an applied voltage of +50 to +70 mV, streptavidin was added to the *cis* compartment and the positive voltage was maintained for 3–11 min. During this time the conductance did not decline. Subsequently, when channels were turned off for as little as 5 s by removing the positive voltage, only a small percentage, if any, reopened upon the reapplication of the positive voltage. These observations indicate that during the prolonged positive voltage stimulus, the biotinylated residue had not been exposed to the *cis* solution for more than a few seconds, since if it had, the conductance would have declined substantially during this period. In other experiments (e.g., Fig. 12 *b*), after the 3–11-min exposure to *cis* streptavidin, streptavidin was also added to the *trans* solution; when the voltage stimulus was switched to –50 mV, the channels remained “locked” in an open state, confirming that the biotinylated residue was indeed on the *trans* side. These results were obtained with all of the biotinylated mutants tested in this way: V490C, K511C, R534C, and even I474C (at pH 8.0, where channels did not open at +50 or +60 mV in the presence of *cis* streptavidin). We therefore can conclude that residues capable of being bound by *trans* streptavidin do not flip back to the *cis* side for any appreciable time while the channels are held open by voltages  $\geq +50$  mV; i.e., they do not slide back and forth across the membrane. We cannot pre-

clude, however, transient movement of these residues from the *trans* solution into the bilayer.

## DISCUSSION

The opening and closing of voltage-gated channels presumably involves conformational changes in the proteins forming them. Using site-directed cysteine mutagenesis followed by cysteine-specific biotinylation of the site, and then monitoring by electrophysiological methods the ability of streptavidin to bind to the chemically attached biotin, we had earlier found that voltage gating of colicin Ia channels was associated with the translocation across the membrane of a segment of at least 31 amino acid residues (Slatin et al., 1994). In the present study we have continued with this approach to map the topology of this channel from residue 454 to 572, a region essentially spanning the entire channel-forming domain from its NH<sub>2</sub> terminus to the beginning of the hydrophobic segment near its COOH terminus. That is, we have determined which residues lie on the *cis* side of the membrane, which on the *trans* side, and which reside within the bilayer in the channel's closed and open states. The model we have arrived at for the closed and open states is shown in Fig. 10; to facilitate its discussion, we start from the COOH-terminal end of the protein and work our way to the NH<sub>2</sub> terminus of the channel-forming domain.

### *Channel Topology*

*The hydrophobic segment (residues 573–612).* We believe that colicin Ia is anchored to the bilayer in both the closed and open states of the channel by a transmembrane helical hairpin formed by the 40 amino acid hydrophobic segment extending from T573 to I612. Although this is a very plausible assumption, the evidence in support of it is mostly indirect (we have recently obtained direct evidence for this assumption [Kienker et al., 1996]), and the orientation of the hairpin in the membrane remains controversial (Lakey et al., 1993; Duché et al., 1994; Song et al., 1991; Shin et al., 1993; Palmer and Merrill, 1994). No data on this are offered from the present study, which is concerned with the region upstream from this hydrophobic segment.

*The COOH-terminal end of the voltage-dependent region: region A (residues 544–572).* From the experiments reported in this paper and previously (Qiu et al., 1994; Slatin et al., 1994) we conclude that region A (544–572) resides on the *cis* side of the membrane in the channel's closed state and that at least part of it lies within the bilayer in the open state. This follows from the failure of closed channels (formed from biotinylated K544C, S547C, I552C, W564C, and E572C in this region) to open in the presence of *cis* streptavidin and

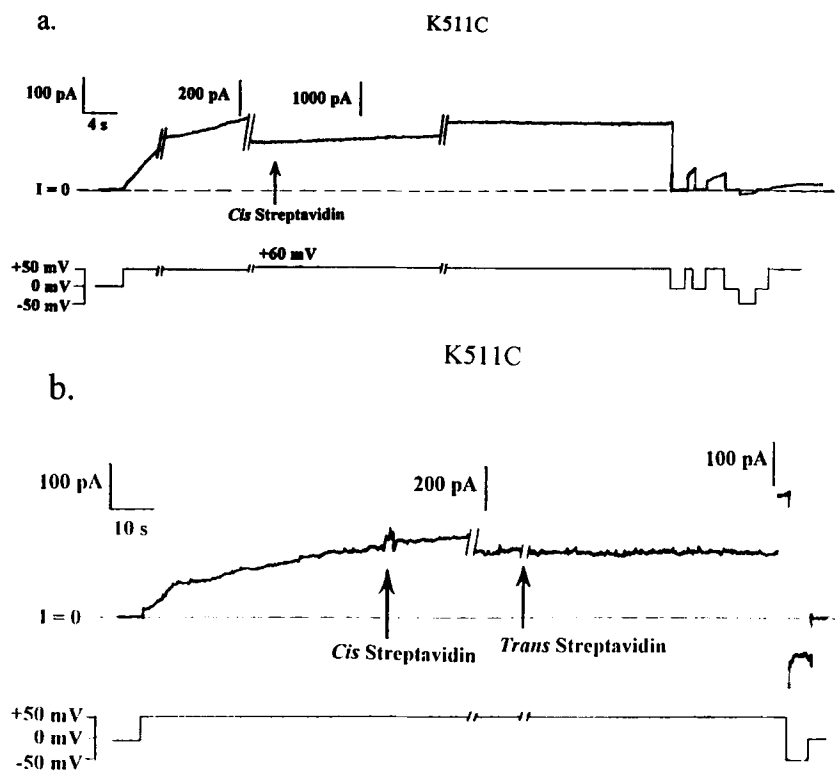


FIGURE 12. Demonstration that residues in region B (in this case residue 511) do not flip back from the *trans* to the *cis* side while the channels are held open by a large ( $\geq +50$ -mV) voltage. (a) Several minutes before the start of the record, 0.2  $\mu$ g of biotinylated K511C (plus 3.5  $\mu$ g of octylglucoside) were added to the *cis* compartment, and the membrane was pulsed several times with positive and negative voltages to confirm normal gating. In the record, the current rose in response to an applied +50- and then +60-mV stimulus. (The first break in the record was 30 s and the second was 1 min. Note the changes in scale.) Streptavidin (10  $\mu$ g) was then added to the *cis* compartment and the applied voltage continued to be held at 60 mV. After the streptavidin was stirred in, the current at +60 mV remained essentially constant over a period of  $\sim 11$  min (including the last break, which was 10 min), indicating that the *cis* streptavidin did not have access to the biotin on residue 511. To confirm that these channels had not lost their sensitivity to streptavidin in the closed state, they were closed by two voltage pulses to 0 mV and finally one to -50 mV; after each of these, the stimulus was returned to +60 mV. All of the

channels had not closed during the short pulses to 0 mV, but we can already see after the second pulse that the response to the subsequent +60 mV was attenuated. The current that developed in response to +60 mV after the pulse to -50 mV was only  $\sim 10\%$  of that at the end of the long, 11-min +60-mV stimulus; that is, 90% of the channel activity had been killed by the *cis* streptavidin. The total time that the channels had been closed by the 0-mV and -50-mV pulses was no more than 5 s, yet in that time, most of the channel activity was killed, whereas no loss of activity occurred over the 11-min period that the channels had been held open at +60 mV. We can conclude from this observation that during the 11-min period, the 511 residue could not have been exposed to the *cis* solution for even a few seconds. (b) Several minutes before the start of the record, 1.2  $\mu$ g of biotinylated K511C (plus 10  $\mu$ g of octylglucoside) were added to the *cis* compartment, and the membrane was pulsed several times with positive and negative voltages to confirm normal gating. In the record, while the current rose in response to a +50-mV stimulus, 10  $\mu$ g of streptavidin were added to the *cis* compartment, and the current eventually reached a steady state early in the first break in the record, which was 5 min. (Note the change in scale.) During the second break (30 s) in the record, 10  $\mu$ g of streptavidin were added to the *trans* solution. When a minute later the voltage was pulsed to -50 mV, a substantial fraction of the current did not turn off, thereby confirming that biotinylated residue K511C was on the *trans* side. The solutions on both sides of the membrane were 100 mM KCl, 5 mM CaCl<sub>2</sub>, 1 mM EDTA, 50 mM HEPES, pH 8.0. Residue K511C was biotinylated with reagent III in Fig. 1, and the sample was purified on the monomeric avidin column, as described in Materials and Methods.

the lack of effect of either *cis* or *trans* streptavidin on channels that are already open.

By itself, the failure of streptavidin from either side to have an effect on channel behavior does not place a given residue within the bilayer in the open state. One can readily imagine steric factors that might prevent a biotinylated residue exposed on either the *cis* or *trans* side of the membrane from binding streptavidin, or that the channel could behave normally even if bound to streptavidin. However, in the context of the accessibility of these residues to *cis* streptavidin in the channel's closed state and the accessibility of residues further upstream to *trans* streptavidin in the open state (see below), we feel that the assignment of at least part of region A to the bilayer interior is compelling. The

homologue in colicin E1 of residue 572 of colicin Ia has been shown to reside near the *cis* mouth of the open channel (Jakes et al., 1990). If residue 572 is comparably situated in the open state of colicin Ia, the protein chain must cross the membrane somewhere between residue 572 on the *cis* side and residue 541 on the *trans* side. We make no prediction about the conformation of this intrabilayer segment. However, it is tempting to suggest that it is an  $\alpha$ -helix, since a portion of region A can be modeled as an amphipathic transmembrane  $\alpha$ -helix. In addition, the secondary structure of the homologous region of colicin A is mostly  $\alpha$ -helical in the crystal structure (Parker et al., 1989).

It should be noted that it was particularly difficult to determine the locations of many residues of region A

in the channel's open and closed states. For example, the mutated residues I552C and W564C were not readily biotinylated. In addition, two other mutated residues in this region (A558C and T571C) could be biotinylated only after denaturing the colicin in urea; the resulting biotinylated mutant protein had lost too much of its channel-forming activity to use for our bilayer experiments. Pending a high resolution crystal structure (see Ghosh et al., 1994), we speculate that these residues may be buried in the protein interior in the water-soluble conformation of colicin Ia.

*The translocated segment: region B (residues 474–541).* From the experiments reported in this paper, and those previously published (Slatin et al., 1994), we conclude that the entire region B, extending from residue 474 to 541, is translocated completely across the membrane from the *cis* to the *trans* side when a channel opens in response to a positive voltage stimulus, and is translocated back from the *trans* to the *cis* side when it closes in response to a negative voltage stimulus. This conclusion follows from the effects of *cis* and *trans* streptavidin on channels formed from eight biotinylated mutants in this region: L474C, V490C, K511C, K524C, R534C, R537C, G540C, and Y541C. The details of the streptavidin effects will be commented upon shortly, but basically, *cis* streptavidin either prevented closed channels from opening or substantially altered their voltage response (in either case demonstrating that, in the closed state, the biotinylated residue was exposed to the *cis* solution), and *trans* streptavidin prevented open channels from closing, thereby demonstrating that in the open state the same residue was exposed to the *trans* solution.

• *The effect of trans streptavidin.* The time required for the *trans* streptavidin effect to develop depended on the location of the biotinylated residue. For channels formed by biotinylated R534C, R537C, G540C, and Y541C (the biotinylated residues closest to the COOH-terminal end of the translocated region), the effect developed slowly over many minutes. The nearer the residue to the COOH terminus, the longer was the time required for a convincing effect to be observed. It is as if these residues are very close to the membrane–solution interface, or even somewhat on the membrane side of this interface. On the other hand, the *trans* streptavidin effect developed rapidly for channels formed by biotinylated L474C, K490C, K511C, and K524C, and was rapidly reversed by DTT or cysteine, as if these residues are well exposed to the *trans* solution.

• *The effect of cis streptavidin.* For channels formed by biotinylated K511C or by biotinylated mutants nearer to the COOH terminus, binding by *cis* streptavidin killed virtually all channel-forming activity. At very large positive voltages, however, a noisy, voltage-dependent conductance was seen (Fig. 9), indicating that it

was still possible to form some sort of channel with streptavidin bound at these sites. For channels formed by biotinylated L474C and V490C, binding by *cis* streptavidin induced a macroscopic conductance not unlike that created by normal channels (see Figs. 7 and 9). As we noted, however, the binding of *cis* streptavidin altered the single-channel conductance and lifetimes of biotinylated L474C (Fig. 8).

*The upstream transmembrane segment of the channel is not uniquely defined.* We conclude that region C (residues 454–466) resides on the *cis* side of the membrane in the channel's closed state, and that it normally lies within the bilayer in the open state, forming part of the upstream transmembrane segment of the channel. This follows from the insensitivity to *cis* and *trans* streptavidin of open channels formed from biotinylated N454C and K466C, and from the effect of *cis* streptavidin on closed channels.

Apparently, however, any one of a number of segments can successfully serve as an alternative upstream transmembrane segment (e.g., Fig. 10). The identity of the transmembrane segment can be altered by the binding of *cis* streptavidin, without destroying the ability of the protein to form a voltage-dependent channel. A segment including residues 454 and 466 normally is the upstream transmembrane segment, but if, for example, residue 474 is forced to remain on the *cis* side by streptavidin, then another segment downstream of residue 474 substitutes for the normal one. This new channel structure is accompanied by a shift in the voltage dependence and a change in single channel conductance. Nevertheless, we make the parsimonious assumption that the streptavidin-bound channels are functioning fundamentally like unbound channels. That is, we assume that whatever (as yet undiscovered) mechanism allows the COOH-terminal domain of colicin Ia to induce an ion-conducting pathway across a lipid bilayer, that same mechanism accounts for the channel-forming ability of the streptavidin-bound channels.

*The overall picture.* The implication of our results is illustrated in the model depicted in Fig. 10. We postulate that, on the downstream side of region B (residues 474–541), region A (residues 544–572) contains an essential transmembrane segment of the open channel. The transmembrane segment on the upstream side of region B is somewhat arbitrary. Thus, in the open state, residues 474 and 490 are normally on the *trans* side of the membrane and residue 454 is in the membrane, probably near the *cis* side, implying that the upstream transmembrane segment lies between residues 454 and 474. Since biotinylated N454C is not accessible to *cis* streptavidin in the channel's open state (Fig. 11), we have not unambiguously identified the NH<sub>2</sub> terminus of the upstream transmembrane segment. (Channels

formed by biotinylated S402C were only subtly affected by *cis* streptavidin and not affected at all by *trans* streptavidin. Since residue 402 is well outside of the channel-forming domain (Cramer et al., 1995), this result serves as a control, showing that, at least somewhere upstream from residue 454, there is a region that remains on the *cis* side when the channel opens.) On the other hand, if residue 474 or 490 is held on the *cis* side by *cis* streptavidin, relatively normal voltage gating still occurs (see Figs. 7 and 9), implying that the upstream transmembrane segment can be formed from residues nearer the COOH-terminal end. (Even the binding of residues 511 or 534 by *cis* streptavidin still allows voltage-dependent gating, albeit highly aberrant [Fig. 9].) Although the channels formed under these conditions are not identical to the normal channel (Fig. 8), the voltage gating is similar enough that one may conclude that the upstream transmembrane segment is not well-defined, and that multiple open states for the channel are possible, corresponding to different upstream transmembrane segments. In the absence of *cis* streptavidin, however, the segment between residues 474 and 534 remains on the *trans* side in the open channel state and does not wander back and forth between the *cis* and *trans* sides (Fig. 12).

#### *Channel Structure and Gating*

Although we have shown how several regions of the colicin Ia molecule move with respect to the membrane during channel gating, three fundamental issues about channel structure and gating remain unresolved. First, there does not appear to be enough protein to form the channel, given that studies of colicins E1 and A indicate that: (a) a channel is formed by one molecule (Jacob et al., 1952; Wendt, 1970; Bruggemann and Kayalar, 1986; Peterson and Cramer, 1987; Slatin, 1988; Levinthal et al., 1991); (b) the pore diameter is at least 9 Å (Raymond et al., 1985; Bullock et al., 1992; Krasilnikov et al., 1995); and (c) colicin fragments as short as 94 residues (corresponding to residues 533–626 of colicin Ia, and thus lacking region C and most of region B) can still form some sort of voltage-gated channels (Liu et al., 1986; Baty et al., 1987). The results presented in this paper exacerbate this problem. In our model of the open channel (Fig. 10), there are only four transmembrane segments, with half the residues of the channel-forming domain residing outside of the membrane. Although changing the upstream transmembrane segment (as by binding *cis* streptavidin at residue 474) clearly influences channel properties, its role in pore formation could be nonessential. In fact, if short colicin fragments have the same membrane topology that we have demonstrated for the whole colicin, it would suggest that a colicin channel can be formed

from only three transmembrane segments (one from region A and two from the hydrophobic region). To make a 9-Å diameter channel would require at least six closely packed  $\alpha$ -helices (Dunker and Zaleske, 1977). Although a less tightly packed structure might form a somewhat larger pore, it is hard to imagine how a 9-Å channel could be formed with only three helices, considering that studies with model amphiphilic peptides (unrelated to colicins) indicate that four transmembrane helices can form a channel of <1-Å diameter (Lear et al., 1988). At this point we have no answer and can only invoke the lipid, “the last refuge of the intellectually bankrupt” (personal condemnation conveyed through O. S. Andersen), as forming part of the channel structure.

If region A indeed contains an essential transmembrane segment of the colicin channel, whereas regions B and C are nonessential, the following interesting question presents itself. If channels can exist in an open or closed state with, for instance, residue 474 held on the *cis* side by streptavidin, why can't they close when this residue is held on the *trans* side by streptavidin? That is, we know there is an alternate open state with residue 474 held on the *cis* side, suggesting that region B is sufficiently “flexible” to reach to the *trans* side and still permit region A to span the membrane. Thus we might expect that with residue 474 held on the *trans* side, region B should be sufficiently long and flexible to allow region A to flip out of the membrane back to the *cis* side, closing the channel. In fact, we observe the opposite, that binding *trans* streptavidin to residue 474 prevents closing. This behavior would be consistent with a kinetic closing pathway in which region C must first move back to the *cis* side, followed by region B, and lastly region A, much like a string being pulled through the membrane from the upstream end. If the return of region B to the *cis* side is blocked by *trans* streptavidin, then region A cannot return either, and so the channel cannot close. (This model suggests that the channel might open via the reverse of this mechanism; i.e., the initial event in channel opening is the insertion of region A, followed by the unravelling of region B through the membrane.) Of course, other models might also explain these results.

Second, the cause of the voltage-dependent gating in this system is not understood. We have shown here that region B (residues 474–541) moves across the membrane with channel opening and closing. There are 15 basic and 8 acidic residues in this region (Mankovich et al., 1986). At neutral pH, they should each contribute a full charge to the voltage dependence of gating, for a net seven positive charges. The charged residues in the upstream (454–474) and downstream (544–572) transmembrane regions should also make partial contributions to the gating charge, in proportion to the fraction

of the transmembrane electric field which they traverse during channel gating; these contributions would probably be small relative to the contribution of the translocated region. The gating charge of colicin Ia has been measured to be three to four charges at neutral pH (Nogueira and Varanda, 1988), considerably less than the expected seven. The discrepancy may arise from any of a number of factors. For example, the complexities of channel gating (e.g., multiple open states) complicate the measurement of gating charge. In addition, we should not rule out the possibility that the ionizable groups actually cross the membrane predominantly as neutral species, even though such species represent a

small fraction of the population. If those were the species that cross the membrane, they would not contribute to the voltage dependence. Thus far, however, we have not identified the voltage sensing elements.

Finally, we have no understanding of how region B, a stretch of at least 68 amino acid residues, is translocated across the membrane. It hardly seems possible, from energetic considerations, that this hydrophilic segment could move through the bilayer proper. In some way, the combination of the hydrophobic helical hairpin with region A must provide a low-energy pathway for region B to cross the membrane. The nature of this pathway remains to be elucidated.

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