Quarterly Reviews of Biophysics **32**, 2 (1999), pp. 189–205 Printed in the United Kingdom



# Channel-forming colicins: translocation (and other deviant behaviour) associated with colicin Ia channel gating

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## 1. Introduction

Colicins are plasmid-encoded proteins, produced by some strains of *E. coli*, that kill other strains lacking the specific immunity protein encoded by the same plasmid. Most of the colicins have a three-domain structure: a central domain that binds to a receptor in the outer membrane of the target cell; an N-terminal domain that interacts with target cell proteins to move the C-terminal domain across the outer membrane and periplasmic space to the inner membrane; and a C-terminal domain that carries the toxic activity. In some colicins the C-terminal domain is an enzyme that kills the cell by entering the cytoplasm and attacking its DNA (e.g. colicin E2), its ribosomal RNA (e.g. colicin E3), or another target (Schaller

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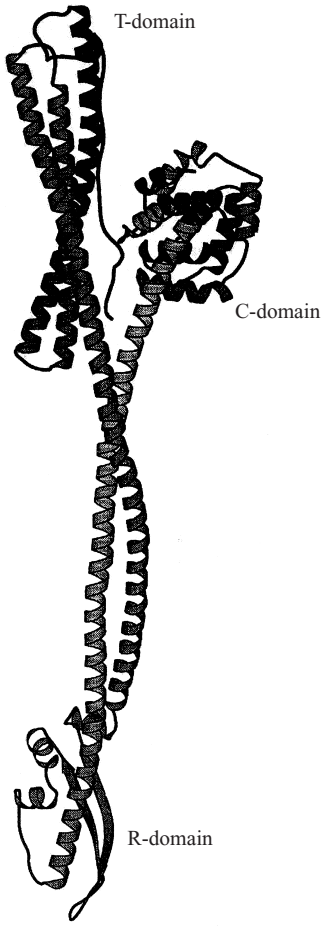


**Fig. 1.** The crystal structure of the water-soluble form of colicin Ia. Note the long α-helices connecting the receptor-binding domain (R) to the N-terminal domain (T) and the channel-forming domain (C). (From Wiener *et al*. 1997.) (Reprinted by permission from *Nature*, **385**, 461–464, copyright 1997 Macmillan Magazines Ltd.)

*et al*. 1982; Ogawa *et al*. 1999). In other colicins, the C-terminal domain forms an ion-conducting channel in the inner membrane that ultimately leads to cell death by allowing essential solutes to leak out of the cell. These colicins, or their isolated C-terminal domains, can also form voltage-dependent channels in planar phospholipid bilayers. (For a review of the E colicins, including enzymatic colicins, see James *et al*. 1996; for a review of channel-forming colicins, see Cramer *et al*. 1995; and for a review of colicin import into *E. coli*, see Lazdunski *et al*. 1998.) The channel-forming colicins are the subject of this review, with particular emphasis on one member of this group, colicin Ia, and the protein translocation associated with the gating of its channel.

Colicin Ia is a water-soluble protein consisting of 626 amino acid residues (Mankovich *et al*. 1986). Its three-domain nature, inferred from functional studies, many of which were done on other channel-forming colicins (Brunden *et al*. 1984; Liu *et al*. 1986; Baty *et al*. 1988; Ghosh *et al*. 1993), is dramatically confirmed by its X-ray crystallographic structure (Wiener *et al*. 1997) (Fig. 1). Two extraordinarily long (∼ 160 Å) α-helices connect the central

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receptor-binding domain to the other two domains in a Y-shaped structure. Thus, with the receptor-binding domain attached to its outer membrane receptor, these helices are long enough to allow the other two domains to reach the plasma membrane, a fact undoubtedly relevant for the function of the channel-forming domain. It is possibly also relevant for the function of the N-terminal domain. There is evidence that, *in vivo*, colicin A spans the space from the outer to the inner membrane during cell killing (Bénédetti *et al*. 1992). The channel-forming domain of colicin Ia comprises the C-terminal 177 residues (450–626) and consists of 10 α-helices (designated C1 through C10) and their connecting loops; this structure is very similar to that of the colicin A, E1, and N channel-forming domains, whose X-ray crystal structures have also been solved (Parker *et al*. 1989, 1992; Elkins *et al*. 1997; Vetter *et al*. 1998). All of the channel-forming colicins have, near their carboxy-terminal end, a stretch of 31–49 uncharged amino acid residues (40 residues in colicin Ia) (Yamada *et al*. 1982; Morlon *et al*. 1983; Mankovich *et al*. 1986; Pugsley, 1987). Within this region, a hydrophobic hairpin is formed by the two helices C8 (residues 580–594) and C9 (residues 597–612) of colicin Ia (Wiener *et al*. 1997). Analogous hydrophobic hairpins occur in colicins A, E1 and N (Parker *et al*. 1989, 1992; Elkins *et al*. 1997; Vetter *et al*. 1998) and can be inferred from the sequences of the other channel-forming colicins. Aside from the hydrophobic hairpin, the colicins are rich in charged amino acid residues.

The structure of a channel-forming protein can be determined by X-ray diffraction, if the protein is crystallized in a 'membrane-like' environment (e.g. porins (Weiss *et al*. 1991; Schirmer *et al*. 1995), the KcsA potassium channel (Doyle *et al*. 1998), and α-hemolysin (Song *et al*. 1996)). In contrast, the colicin structures obtained to date correspond to the water-soluble form of the molecules. Colicins, and components of other toxins such as diphtheria toxin's T-domain (Choe *et al*. 1992), must undergo a radical change in conformation to form a channel. Nonetheless, the crystal structure of colicin Ia in its water-soluble form is an invaluable guide to our speculations about the structure of the membrane-inserted channel. We assume that the channel-forming domain maintains its predominantly α-helical character through all its conformational changes, although the exact lengths of its helices may be somewhat different in the membrane than in solution, as has been shown for colicin E1 in its membrane-associated closed state (Zakharov *et al*. 1998).

## 2. Channel properties

### 2.1 Voltage-dependent gating

When reconstituted into planar phospholipid bilayer membranes,[1] all of the channel-forming colicins form channels that turn on (i.e. open) at positive voltages, and turn off (i.e. close) at negative voltages. (The voltage is the potential of the *cis* compartment (the side to which colicin was added) with respect to the opposite *trans* compartment, whose potential is taken as zero.) The kinetics and voltage sensitivity of channel gating not only differ greatly among the colicins but also can change dramatically for a given colicin as a function of pH (see, for example, Raymond *et al*. 1986; Collarini *et al*. 1987). Colicin Ia can form voltage-dependent channels in planar bilayers over a range of pH from 4·0 to 9·0 (Nogueira & Varanda, 1988; Ghosh *et al*. 1993; Kienker *et al*. 1997); at sufficiently low pH ($< 5$), it inserts into lipid

[1] In this review, we mostly confine our discussion of colicin channel behaviour to that observed in planar bilayers; results in lipid vesicles can be found in Cramer *et al*. (1992).

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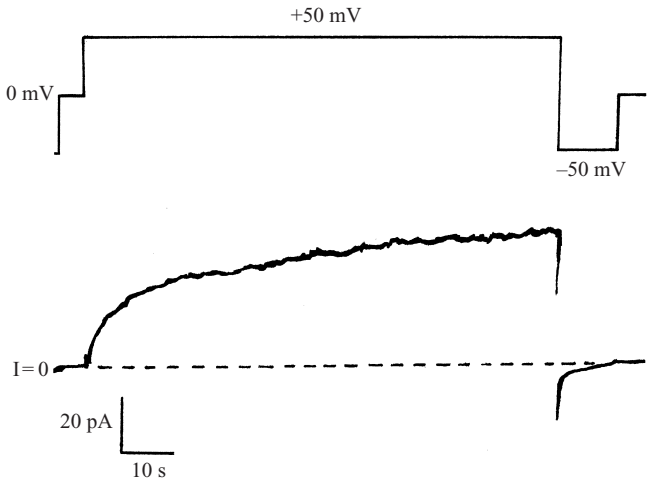


**Fig. 2.** Macroscopic (many channels) current record of a colicin Ia-treated membrane. Note that in response to the +50 mV stimulus, the current initially rises with approximately $[1-\exp(-t/\tau)]$ kinetics followed by a linear increase; when the voltage is switched to −50 mV, the current rapidly declines to zero. (Modified from Qiu *et al*. 1994.)

vesicles at zero voltage (Mel & Stroud, 1993). The number of channels incorporated into planar bilayers can be increased by the addition of octyl glucoside to the colicin preparation (Bullock & Cohen, 1986).

After colicin Ia has been added to the *cis* compartment (to a concentration of $\sim 0{\cdot}1$ to 1 $\mu$g/ml), when a positive voltage of sufficient magnitude ($\geqslant 20$ mV) is applied across the membrane, the current rises with approximately $[1-\exp(-t/\tau)]$ kinetics (where $\tau$ is of the order of a fraction of a second to a few seconds), followed by a slow linear increase with time (Nogueira & Varanda, 1988). The slow linear current rise at positive voltages reflects the insertion of new channels into the membrane; if the sign of the voltage is reversed, the current rapidly declines to a low value comparable to that of an unmodified membrane (Qiu *et al*. 1994) (Fig. 2). To attempt to quantify the voltage dependence of the channels that are already incorporated in the membrane, one can take for the steady-state current at a given voltage the maximum current level achieved prior to the beginning of the linear rise. This gives an estimate of steady-state conductance $G$ (current divided by voltage) as a function of voltage ($V$), that is reasonably well fit by the Boltzmann relationship:

$$G = \frac{G_{\max}}{1 + e^{\dfrac{-nF(V - V_o)}{RT}}}, \tag{1}$$

where $G_{\max}$ is the maximum membrane conductance (approached at large values of $V$, when all of the channels are open), $F$ is the Faraday, $R$ is the gas constant, $T$ is the absolute temperature ($RT/F = 25{\cdot}5$ mV at 23 °C), $V_o$ is the voltage at which half the channels are open, and $n$ is the effective gating charge. The value of $n$ is pH dependent, decreasing from about 6 at pH 4·5 to about 3 at pH 8·2; over this same pH range, $V_o$ increases from 25 to 38 mV, and the product, $nFV_o$, is roughly constant at around 3 kcal/mol (Nogueira & Varanda, 1988).

The Boltzmann relation given by equation (1) applies to a channel with two states, closed

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and open, where the free energy difference between those states is the sum of the 'chemical', non-voltage-dependent energy ($-nFV_o$) and the electrical energy ($nFV$). The simplest interpretation of the voltage dependence is that as the channel opens, a net $n$ positive charges cross the membrane from the *cis* to the *trans* side. (More complex interpretations, involving movement of negative charges from the *trans* to the *cis* side, or a net charge larger than $n$ moving through a fraction of the potential difference $V$, can equally well account for the voltage dependence.) Unfortunately, as we shall see later, colicin Ia cannot be viewed as forming a simple, two-state system, and the value of $n$ obtained from equation (1) cannot be related in any straightforward way to what we now know moves across the membrane when the channels open.

One of the complications, for example, that arises in colicin channel gating is the phenomenon of inactivation seen at large positive voltages; that is, after reaching a peak, the current declines to a lower level. This behaviour is strikingly observed with colicin E1, where associated with the inactivated state, some channels become capable of being turned on at negative voltages and turned off at positive voltages, as if the channel-forming region of the molecule has been translocated across the membrane and reinserted from the opposite side (Slatin *et al*. 1986). The contribution of inactivation to the 'steady-state' conductance seen at lower positive voltages introduces at least one other closed state to the system.

### 2.2 Ion permeability

The selectivity of a channel for different ionic species can be determined from the reversal potential (the voltage at which there is no net current through the membrane) when there is an ionic concentration gradient across the membrane. If the channel is permeable to only one type of ion, the reversal potential should equal the equilibrium (Nernst) potential for that ion. Colicin channels are permeable to both cations and anions. This is revealed by reversal potentials intermediate between the equilibrium potentials of the cation and the anion in a single-salt gradient experiment (Schein *et al*. 1978; Raymond *et al*. 1985). The relative cation to anion permeability of the colicin channels is a function of the particular ion pair, the pH of the solutions, and the lipid composition of the membrane (see below).

#### 2.2.1 Selectivity between potassium and chloride

In membranes formed from asolectin, which contains about 20% negatively charged phospholipids, colicin channels are more permeable to $K^+$ than to $Cl^-$ at pH $\geqslant$ 4·5; the higher the pH, the greater the cation selectivity. Thus, for a colicin Ia-treated membrane separating 1 M KCl from 0·1 M KCl, the reversal potential is 32 mV (preferentially cation selective) at pH 9·0 and close to 0 mV (nonselective) at pH 4·5 (P. Kienker, unpublished results). Colicin E1 channels become anion selective below pH 5 in asolectin membranes, and below pH 8 in membranes formed from phospholipids with no net charge (Raymond *et al*. 1985).

#### 2.2.2 Permeability to large cations and large anions

By measuring the reversal potential for a single-salt gradient of a large cation and small anion (e.g. glucosammonium chloride) or a large anion and a small cation (e.g. sodium glucuronate), one can determine if the large ion is permeant through the channel. (A

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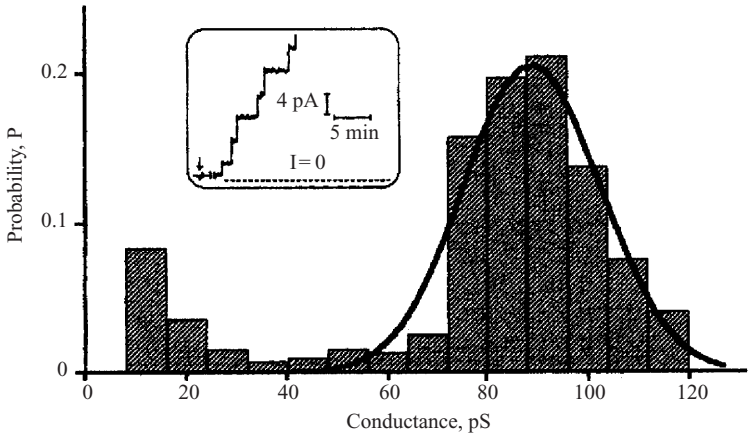


**Fig. 3.** Distribution of single channel conductances of colicin Ia. The inset is a record of single channel current vs. time (at +50 mV). (From Krasilnikov *et al.* 1998. Reprinted by permission from *J. Membrane Biology*, **161**, 83–92, copyright 1998, Springer-Verlag.)

significant deviation of the reversal potential from the equilibrium potential for the small ion would demonstrate that the large ion is permeant.) Experiments on colicins E1, A and Ib have shown that their channels are permeable to ions as large as glucosammonium$^+$ and NAD$^-$, indicating a channel diameter of at least 8 Å (Raymond *et al.* 1985); more extensive experiments on colicin E1 gave a lower limit of about 9 Å (Bullock *et al.* 1992; Bullock & Kolen, 1995). Experiments on colicin Ia are roughly consistent with these estimates. For colicin Ia-treated membranes separating 1 M from 0·1 M bis–tris propane chloride at pH 6·3, the reversal potential was 29 mV (anion selective) (P. K. Kienker, unpublished results). The equilibrium potential for chloride under these conditions was measured as 44 mV, using the chloride-selective carrier, $(C_6F_5)_2Hg$ (Andersen *et al.* 1976). Thus, the channel is permeable to bis-tris propane, indicating a diameter of at least 7·5 Å at the narrowest point. Because the test molecules used for all of the colicin channel experiments are not spherical and have some flexibility, exact values cannot be placed on the channel diameter from these measurements, but simply in terms of the ions that are permeant, all of the colicin channels are large compared to the highly selective channels found in neuronal plasma membranes.

## 2.3 Single-channel characteristics

One of the major aims of the investigation of colicin channels is to arrive at a model for their structure, from which one may hope to deduce physico-chemical rationales for their ion permeability, voltage gating, and the massive protein translocation (see below) associated with their gating. An indication that these channels may not be of the same ilk as the physiological channels found in cell membranes is the distribution of their single-channel conductances. Whereas the traditionally studied channels, such as the voltage-dependent sodium, potassium, and calcium channels have fairly well defined single-channel conductances (Oral Tradition), the distribution of single-channel conductances for the colicins is rather broad. For the colicin Ia channel in 1·77 M KCl at pH 7, the most frequently observed conductances are spread out between 60 and 120 pS, with significant sightings of channels of much smaller conductances (Krasilnikov *et al.* 1998) (Fig. 3). Conductances are pH dependent,

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being about two-fold smaller at pH 5 than at pH 8 (Nogueira & Varanda, 1988). (The dispersion of single-channel conductances is much greater for colicin E1 (Bishop *et al*. 1986), making it difficult even to talk about a predominant range of values for that colicin.) The small magnitude of the single-channel conductance ($\sim$ 7 pS in 0·1 M KCl (Schein *et al*. 1978)) is somewhat surprising, given the large diameter of the channel lumen. As has been pointed out, however, factors other than size can play a major role in determining channel conductance, as witness the double-length nystatin channel, which has a diameter of 8 Å but a conductance of only 1 pS in 2 M KCl (Kleinberg & Finkelstein, 1984). The voltage gating of colicin Ia single channels is in qualitative agreement with the voltage dependence measured in membranes containing many channels. That is, channels are mostly open at voltages much greater than $V_o$, flicker on and off at voltages around $V_o$, and are mostly closed at voltages much lower than $V_o$ (Nogueira & Varanda, 1988).

In the previous section we described the sizing of colicin channels from macroscopic reversal potential measurements with gradients of large cations and large anions across colicin-treated membranes. An independent determination of channel size for colicin Ia has been made from the effect of nonelectrolytes on single-channel conductance, the idea being that a nonelectrolyte that is small enough to enter the channel will decrease single-channel conductance, whereas one that is too large to enter will have no effect (after accounting for any changes in the conductivity of the bulk solution). Using this approach, Krasilnikov *et al*. (1998) concluded that the channel is hourglass-shaped, with an entrance of $\sim$ 18 Å diameter on the *cis* side, $\sim$ 10 Å on the *trans* entrance, and a constriction of $\sim$ 7 Å between the entrances, but nearer the *trans* entrance. This last number is in rough agreement with the large-ion sizing studies referred to earlier.

### 2.4 Molecularity of the channel

As we shall see in the following part of this article, the colicin Ia molecule contributes only four transmembrane segments to the channel. This, plus the large diameter of the channel, would lead any rational person to conclude that the channel is a multimer. Every study, however, that has been directed at determining the molecularity of any of the colicin channels is consistent with its being a monomer (Jacob *et al*. 1952; Wendt, 1970; Bruggemann & Kayalar, 1986; Peterson & Cramer, 1987; Slatin, 1988; Levinthal *et al*. 1991). Although caveats can be attached to each of these determinations, it is striking that none of these studies has indicated that the colicin channel is multimeric. We shall return to this point after our considerations of transmembrane segments and protein translocation.

## 3. Colicin Ia channel topology and protein translocation

### 3.1 Channels formed by whole colicin Ia

Studies of the effects of proteases on colicin E1 channels suggested that parts of the colicin molecule move into and across the membrane in conjunction with voltage gating (Raymond *et al*. 1986; Slatin *et al*. 1986). In view of these results and in the absence of a crystal structure for the membrane-associated form of colicin Ia, we developed a method that can report on

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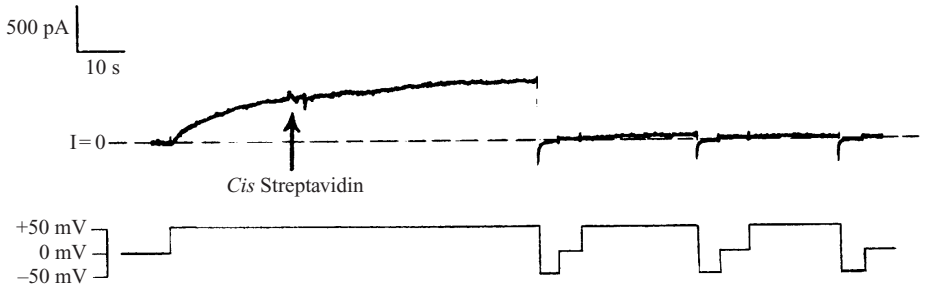


**Fig. 4.** The killing by *cis* streptavidin of the conductance induced by the biotinylated colicin Ia mutant K544C. Note that in the open state the channels are not affected by *cis* streptavidin, but once they have been turned off by the −50 mV pulse, they do not re-open. Thus residue 544 is exposed to the *cis* solution in the channel's closed state, but not in its open state. (From Qiu *et al*. 1996). (Reproduced from *The Journal of General Physiology*, 1996, **107**, 313–328, copyright 1996, The Rockefeller University Press.)

the position of a given amino acid residue with respect to the membrane. This method can indicate whether that residue is exposed on the *cis* or *trans* side, or is buried within the membrane, in the closed and open states of the channel (Qiu *et al*. 1994, 1996; Slatin *et al*. 1994). We used the high-affinity binding of biotin and streptavidin ($K_d \sim 10^{-15}$ M) as the means to detect a particular amino acid residue on one side of the membrane or the other. First, a unique cysteine residue was introduced into the sequence of colicin Ia. (Wild-type colicin Ia has no cysteine (Mankovich *et al*. 1986).) Biotin was attached chemically to the cysteine side chain and the biotinylated colicin Ia was added to the *cis* side of a planar bilayer to form channels. We then added streptavidin, a water-soluble biotin-binding protein, to either the *cis* or *trans* side of the membrane, and monitored its effect (if any) on the behavior of the channels. We reasoned that if a residue moves into or across the membrane when the channel goes from the closed to the open state, anchoring it on the *cis* side with streptavidin should prevent channel opening. Similarly, if a residue moves all the way across the membrane in conjunction with channel opening, it should be possible to anchor it on the *trans* side and prevent channel closing. A residue that moves into the membrane (but not all the way across it) when the channel opens should become inaccessible to streptavidin from both the *cis* and *trans* sides of the membrane. An illustration of the effect of *cis* streptavidin on channel opening is shown in Fig. 4. The effect of *trans* streptavidin on channel closure, at both the macroscopic and single-channel level, is shown in Fig. 5.

### 3.1.1 General channel topology

Figure 6 summarizes the membrane topology of colicin Ia in the closed and open states of the channel, as revealed by this approach (Slatin *et al*. 1994; Qiu *et al*. 1996). In the closed state (Fig. 6*a*), the hydrophobic α-helical hairpin near the C-terminal end of the molecule (residues 580–612) associates with the membrane in a voltage-independent manner. The hairpin is shown in a transmembrane orientation in Fig. 6*a*, but it is in equilibrium with an orientation parallel to the membrane surface on the *cis* side, which, in turn, is in equilibrium with free colicin Ia in solution (Kienker *et al*. 1997) (Fig. 7). All of the residues upstream from this hydrophobic hairpin are on the *cis* side of the membrane. In the open state (Fig. 6*b*), two additional segments, one between residues 541 and 577, corresponding approximately to

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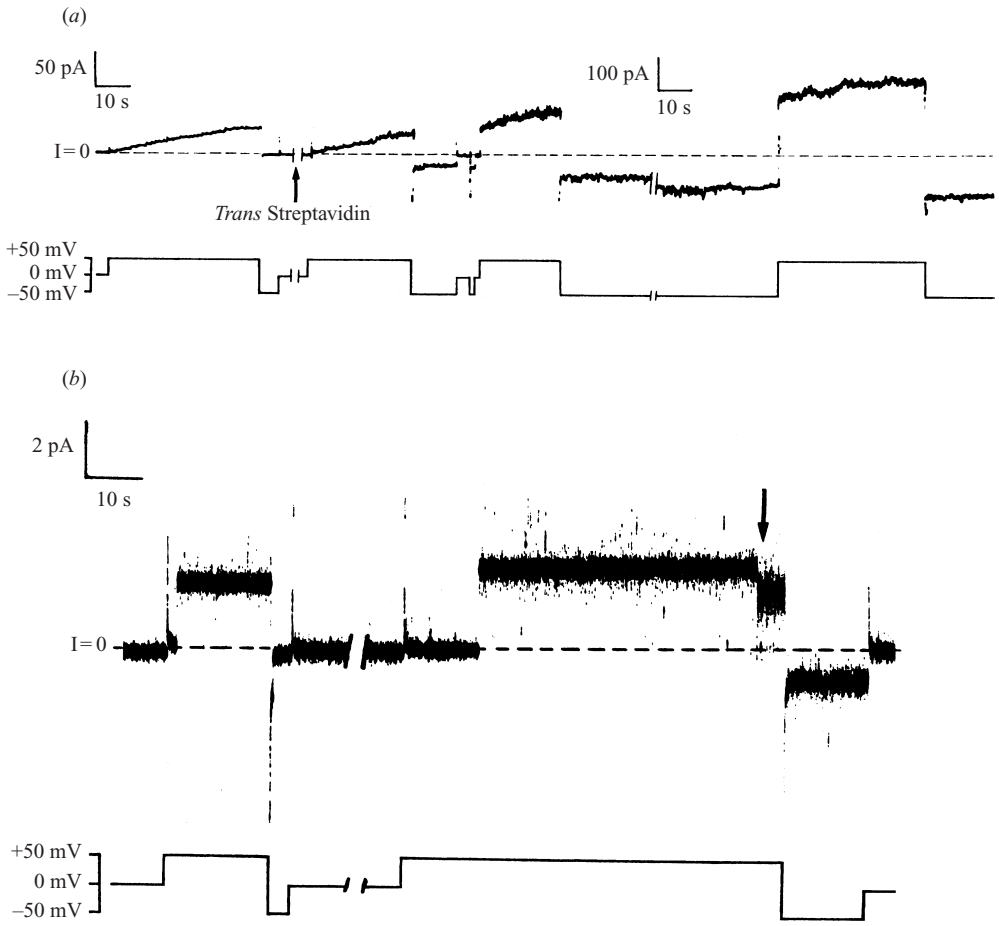


**Fig. 5.** Effect of *trans* streptavidin on the channels formed by the biotinylated colicin Ia mutant R534C. In the macroscopic record (*a*), before the addition of *trans* streptavidin, current rapidly went to zero when the voltage was switched from +50 to −50 mV; after *trans* streptavidin addition, over time, more and more of the current failed to decline at −50 mV, as the biotinylated channels were bound by streptavidin. In the single-channel record (*b*), streptavidin was already present in the *trans* compartment. The arrow marks the putative streptavidin binding event. Note that the conductance of the channel drops at this point. Prior to that event, the channel rapidly and completely closed when the voltage was switched from +50 to −50 mV; after the event, the channel did not close. (From Slatin *et al.* 1994.) (Reprinted by permission from *Nature*, **371**, 158–161, copyright 1994, Macmillan Magazines Ltd.)

helices C6 and C7 of the Ia crystal structure[2], and one from slightly before residue 454 to near residue 474, are inserted into the membrane, and the entire region between residues 474 and 541 inclusive is on the *trans* side of the membrane. (This translocated segment corresponds to helices C2–C5 of the crystal structure.) When the channel closes, all of these residues move back to the *cis* side. Thus, the voltage-dependent opening and closing of the channel is accompanied by the insertion into the membrane and de-insertion out of the membrane of two segments of about 20–30 amino acids each, and by the translocation of at least 68 residues (474–541) back and forth across the membrane.

[2] Based on experiments using biotinylated mutant 577C and streptavidin, it appears that residue 577 remains on the *cis* side of the membrane in both the closed and open states of the channel (K. Jakes & P. Kienker, unpublished results).

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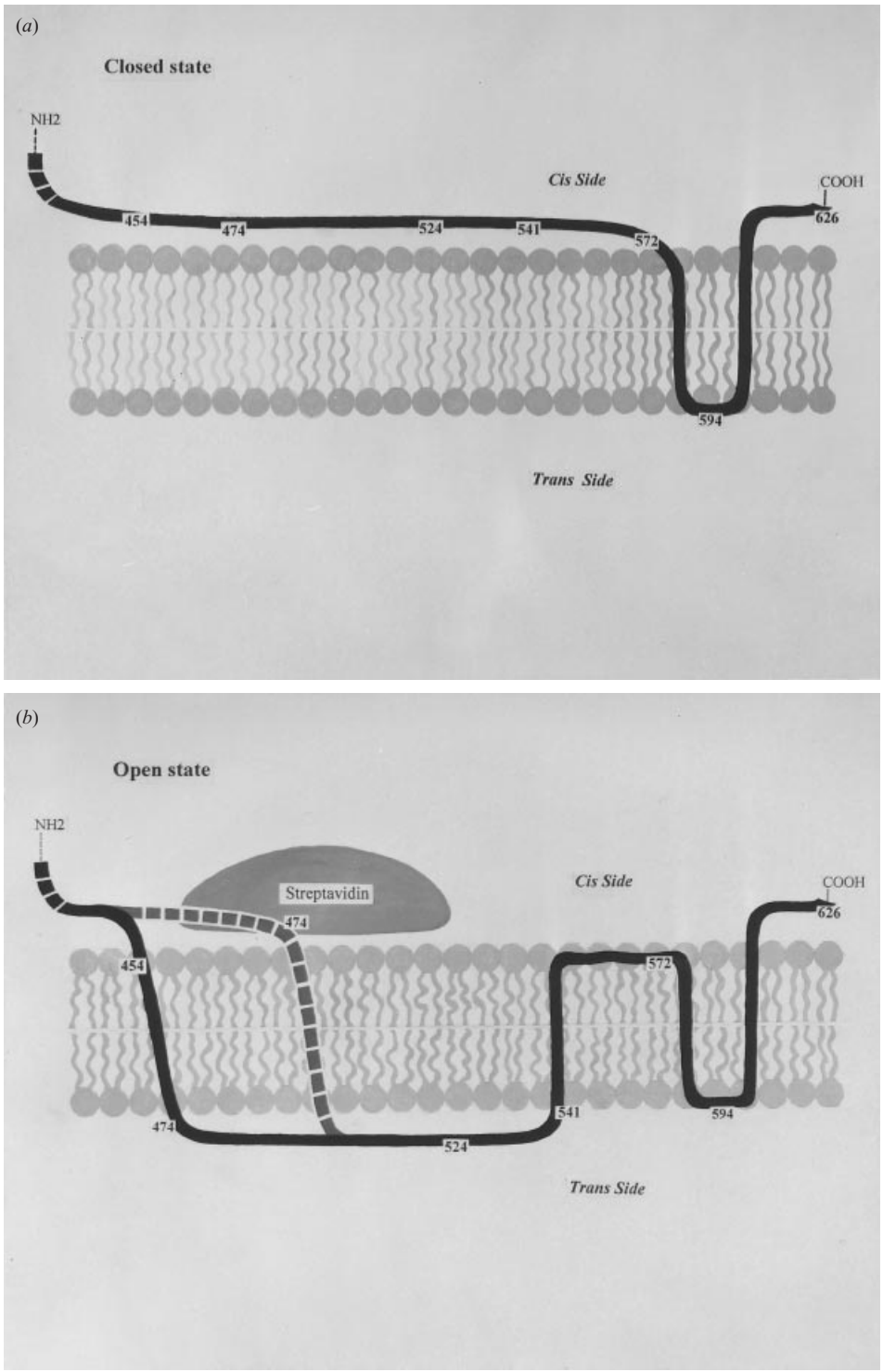


**Fig. 6.** Model of the membrane topology of the colicin Ia channel in its closed and open states. The dashed portion in the open state drawing shows that when residue 474, which is normally translocated to the *trans* side, is held on the *cis* side by streptavidin, an alternative region becomes the upstream bilayer-inserted segment. (From Qiu *et al*. 1996.) (Reproduced from *The Journal of General Physiology*, 1996, **107**, 313–328, copyright 1996, The Rockefeller University Press.)

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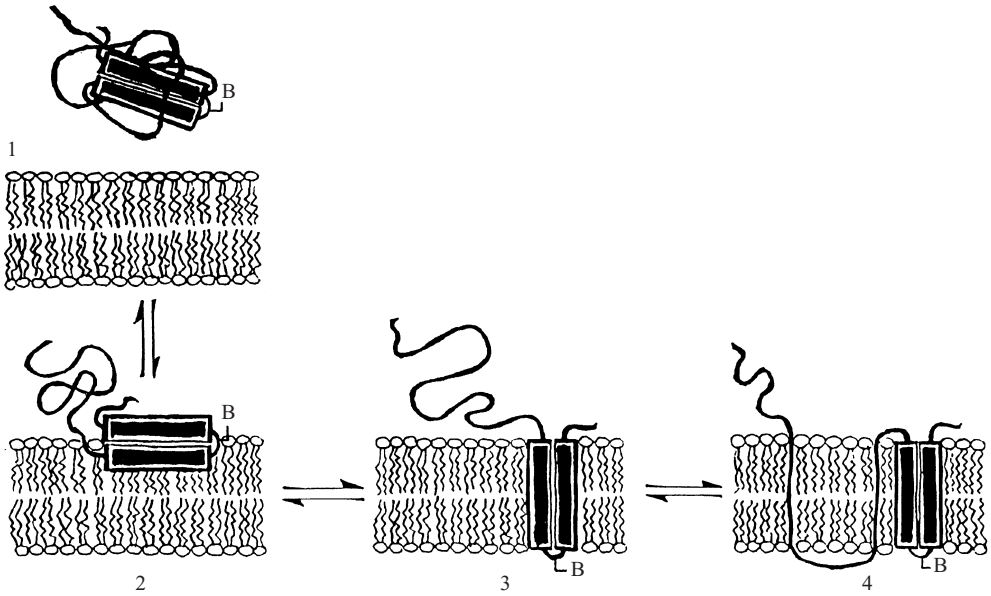


**Fig. 7.** Model of the orientation of the colicin Ia hydrophobic hairpin (represented as two bars connected by a loop) with respect to the membrane. The rest of the molecule is shown as a wiggly line. Panel 4 represents the open state of the channel, with its additional two transmembrane segments and translocated region. The transitions between states 3 and 4 are the only voltage-dependent transitions. Panel 2 shows the hydrophobic loop oriented parallel to the membrane, and panel 1 shows the colicin Ia free in solution. (From Kienker *et al*. 1997.) (Reproduced from *J. Membrane Biology* **157**, 27–37, 1997, copyright 1997, Springer-Verlag).

### 3.1.2 The translocated region

The segment of some 68 residues that is translocated across the membrane in association with channel gating is very hydrophilic, containing 15 basic and 8 acidic residues. There is nothing obvious about its composition to indicate that it is particularly well suited for membrane translocation; this suggests that the translocation mechanism, whatever it is, may be able to translocate an arbitrary amino-acid sequence.

Consistent with this suggestion was the finding that if either the 12CA5 hemagglutinin antigen (YPYDVPDYA) or the FLAG antigen (DYKDDDDK) was inserted into the middle of the translocated segment, the entire segment, along with the added sequence, was still translocated (Jakes *et al*. 1998). It is particularly striking that the high density of charges in the FLAG antigen (seven of the eight residues are charged) was not a major impediment to translocation. It therefore appears that the colicin Ia translocation apparatus is capable of translocating 'any' peptide sequence.

### 3.1.3 The nonuniqueness of the upstream membrane-inserted segment

Given the topology depicted in Fig. 6, one would expect that if a residue that lies on the *trans* side of the membrane in the open channel state (e.g. residue 474) were held on the *cis* side by streptavidin, the channel would fail to open. Surprisingly, this is not the case. If a residue between 474 and 534, that normally is translocated to the *trans* side, is forced to remain on

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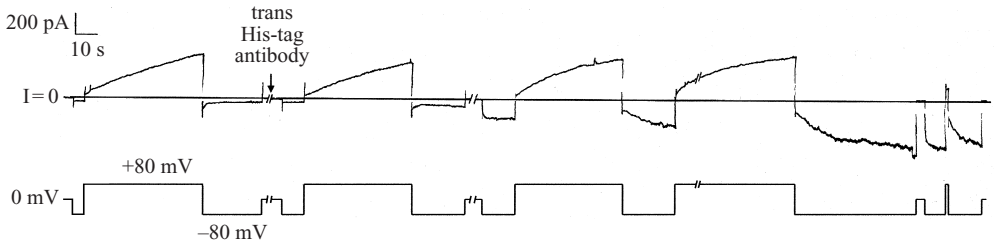


**Fig. 8.** The N-terminal end of the colicin Ia channel-forming domain peptide (residues 453–626) is on the *trans* side in the open state of the channel. When antibody to the N-terminal hexahistidine tag is added to the *trans* solution, the voltage gating of channels formed by the channel-forming domain peptide is reversed, with channels now turning on at negative voltages. That the channels have apparently reversed their orientation in the membrane is not without interest, but the main point for our present purposes is that clearly the N-terminal end of the molecule is on the *trans* side of the membrane in the channel's open state (P. K. Kienker, K. S. Jakes and A. Finkelstein, unpublished).

the *cis* side, a channel is still formed, albeit with a different voltage dependence and single-channel conductance (Qiu *et al*. 1996). Thus, although the normal upstream transmembrane segment includes residues 454 and 466, any one of a number of segments can serve as an alternative. (An example is illustrated in Fig. 6 when residue 474 is held on the *cis* side.) Although the channels formed under these conditions are not identical to the 'normal' channels, it is reasonable to assume that their structure still retains, more or less, the original downstream three transmembrane segments in Fig. 6. In fact, as we shall see in the next section, the isolated colicin Ia channel-forming domain forms channels that have only these three transmembrane segments; these channels have the same qualitative voltage dependence as that of native channels.

### 3.2 Channels formed by the C-terminal domain of colicin Ia

We have just seen that alternative channels and channel structures can be obtained depending on which residues are held (in our case by streptavidin) on the *cis* side of the membrane. What normally holds the residues (near 454) on the *cis* side in channels formed by whole colicin Ia? Is something upstream of the channel-forming domain functioning like our artificially added streptavidin, to arrest translocation at a particular stage? To address this question, we investigated the properties and noumena of channels made by the C-terminal domain alone (residues 453–626). We sought, by biotinylating a cysteine residue introduced at 453 and then adding streptavidin to the *trans* solution, to determine if the N-terminal end of this domain is translocated to the *trans* side. We anticipated that if it is, then grabbing it with *trans* streptavidin should prevent channel turn-off, as observed in analogous experiments with biotinylated residues in the translocated segment of whole colicin Ia. We found that the N-terminal end of the channel-forming domain was indeed translocated to the *trans* side; however, the effect of *trans* streptavidin was not to prevent channel turn-off, but rather to reverse the orientation of the channel in the membrane, so that channels now turned on at negative voltages and off at positive voltages. Comparable results were obtained when antibody to a hexahistidine tag at the N-terminal end was added to the *trans* side (Fig. 8) (Kienker *et al*. 1999).

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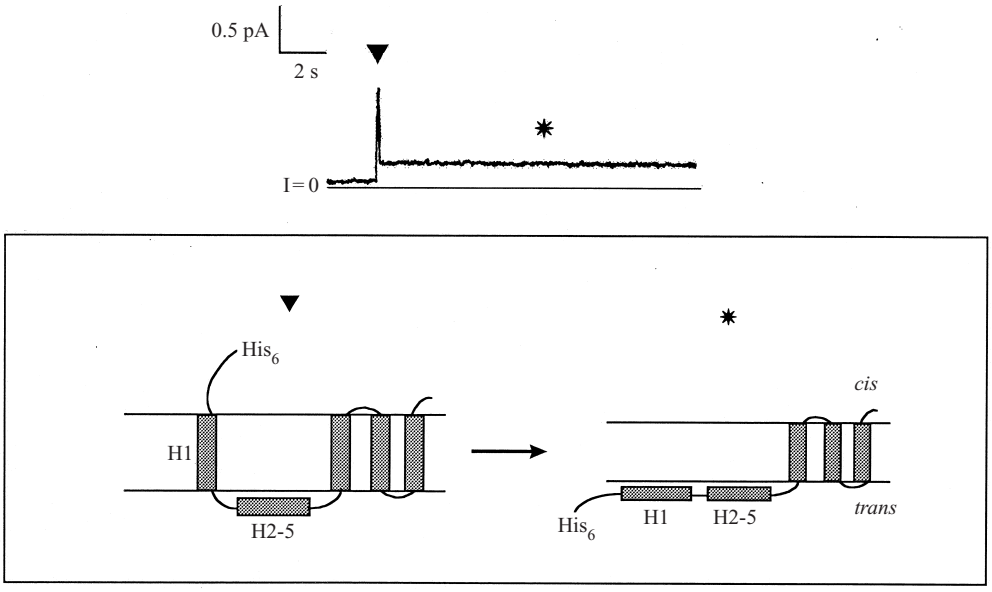


**Fig. 9.** Single channel formed by the colicin Ia channel-forming peptide (residues 453–626). The channel initially opens to a larger value (indicated by the ▼) and then drops to a smaller value (✱). The inset cartoon depicts our model of the transmembrane topology associated with these two open-channel states. The larger channel conductance is associated with the channel formed by four transmembrane segments. The smaller channel conductance occurs when the part of helix 1 in the channel-forming domain (H1) (along with the N-terminal hexahistidine tag ($His_6$) present in our construct) is translocated to the *trans* side, thereby creating a channel with only three transmembrane segments. Helices 2–5 (H2–5) are on the *trans* side in both open states (P. K. Kienker, K. S. Jakes and A. Finkelstein, unpublished).

The ability of the channel-forming domain to reorient in the membrane when its translocated residues are held on the *trans* side by streptavidin or by an antibody is of interest in its own right. The important point for our present purposes, however, is that, in the absence of the rest of the colicin Ia molecule, all of the channel-forming domain upstream of residue 541 is translocated to the *trans* side. Thus, the resulting channel is formed by only three transmembrane segments: two contributed by the C-terminal hydrophobic hairpin, and one by the amphipathic segment bounded (approximately) by residues 541 and 577. The transition from the 'normal' channel formed from four transmembrane segments to the one formed from three segments is seen at the single-channel level (P. Kienker *et al.* 2000). The channel initially opens at a higher conductance (corresponding presumably to the 'normal' structure formed when all four segments are inserted in the membrane), and then drops to the lower conductance of the channel formed by three inserted segments, as the N-terminal segment moves out of the membrane into the *trans* solution (Fig. 9). Differences in the characteristics of channels formed by whole colicins E1 and A from those formed by their corresponding channel-forming fragments have been noted (Raymond *et al.* 1986; Collarini *et al.* 1987), and probably arise for the same reason as described here for colicin Ia. Differences between the channels formed by whole colicin Ia and its C-terminal fragment at low pH have also been reported (Ghosh *et al.* 1993).

In most of the experiments with the channel-forming domain of colicin Ia, there was a hexa-histidine tag at its N-terminal end which was also translocated to the *trans* side, even at

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pH 4·5 (P. K. Kienker *et al*. unpublished observations). At that pH, at least five of the six histidine side chains are charged 98% of the time (Patchornik *et al*. 1957). Because a group of five or six positively charged residues can be translocated, and we previously found with the FLAG antigen that four negatively charged residues in a row can be translocated, what in the upstream portion of whole colicin Ia prevents the translocation of residue 453? We see nothing remarkable in the primary sequence of colicin Ia that could act as an anchor on the *cis* side. We postulate that it is an aspect of the tertiary structure that stops the translocation: for instance, the intertwining of the two very long $\alpha$-helices, or an interaction of the N-terminal domain with the membrane (Fig. 1). If this is the case, one can imagine that there is some flexibility in the boundaries of the membrane-inserted segment at the N-terminal end of the channel-forming domain. This flexibility may account for the dispersion in single-channel conductances mentioned earlier in this article. That is, multiple open states of the channel may normally be present, corresponding to different upstream transmembrane segments.

## 4. Concluding remarks

It appears established from the preceding account that the basic channel element and the translocation machinery are contained in the C-terminal portion of the channel-forming domain, residues 542–626 (helices C6–C10, approximately), which contributes three transmembrane segments. This raises three major questions that are at present unanswered (although hopefully not unanswerable). First, how can a large-diameter channel be made from only four transmembrane segments (in the case of whole colicin Ia) or any channel at all from only three (in the case of the channel-forming fragment)? This would pose no difficulty if the channel were multimeric but, as we mentioned earlier, there is no evidence for this, and, in fact, all of the evidence indicates that the channel is a monomer. In desperation one can invoke the lipids as contributing to the channel structure, although what that structure might be is hypothetical at best.

Second, what accounts for the voltage-dependent gating? There are certainly enough charges moved into and across the membrane to give rise to voltage dependence. The problem is that there are too many charges. As we noted earlier, the translocated region contains 15 basic and 8 acidic residues. Even at high pH ($\sim$ pH 8), this would yield at least 7 gating charges, not counting the net positive charge contained in the membrane-spanning segments. Yet at pH 8, the apparent gating charge is only 3 (Nogueira & Varanda, 1988). Moreover, even after the net charge of the translocated region was converted from $+7$ to $-1$, in the colicin Ia mutant K485N-K486E-R488E-K506N-K507E-S594C, the channels retained the same qualitative voltage dependence, turning on at positive voltages and off at negative voltages (K. S. Jakes & P. K. Kienker, unpublished results). In an attempt to generate a more stable system, we biotinylated colicin mutants at residue S594C, at the tip of the hydrophobic hairpin, and anchored the colicin in the membrane with *trans* streptavidin. This did not solve the problem: the gating charge measured for the mutant S594C was only about 2, independent of pH (Kienker *et al*. 1997); a mutant with three fewer net charges (K485N-K486E-S594C) did not have a significantly different gating charge (P. K. Kienker, unpublished results). Examination of anchored colicin mutants at the single-channel level revealed a complex array of conductance states. The transitions between the predominant 'open' and 'closed' states exhibited more reasonable voltage dependences for some of the

mutants, with a gating charge of 8–9 for S594C, and 5–6 for K485N-K486E-S594C (P. K. Kienker, unpublished results), but a general pattern relating gating charge to the charge of the translocated region has not emerged. The complexities of the gating scheme (for instance, the multiple open states) may make it difficult to correctly measure the voltage dependence. Alternatively, if some of the supposedly charged residues actually cross the membrane in an uncharged form, the voltage dependence would be weaker than expected.

The third major question is the mechanism of translocation. All of the translocation machinery is apparently built into the 85 C-terminal residues (542–626) of the channel-forming domain, which contribute three transmembrane segments. Somehow the interaction of these segments with the bilayer lipids creates a polar pathway for upstream residues to pass through, but the nature and structure of that pathway is at present not known. An analogous, and equally obscure, situation exists with diphtheria toxin, for which a channel-forming region of 114 residues contains the machinery to translocate the upstream 264 residues (Senzel *et al*. 1998; Oh *et al*. 1999). In both the colicin Ia and diphtheria toxin cases, the role of the channel *per se* in the translocation mechanism is unclear. For diphtheria toxin, the channel may be a 'discarded wrapper' not directly related to the translocation of its toxic N-terminal domain (Misler, 1984). Similarly, for colicin Ia, the translocation may be an epiphenomenon not directly related to the channel. The mechanism of translocation operating in colicin Ia, whatever it turns out to be, may be applicable to a number of toxins (besides diphtheria toxin) which contain a channel-forming domain essential for translocation of the catalytic domain to the cytosol. Examples of these include tetanus and botulinum neurotoxins (Hoch *et al*. 1985) and the adenylate cyclase toxin from *Bordetella pertussis* (Szabo *et al*. 1994).

## 5. Acknowledgement

The research on translocation reviewed here was supported by NIH Grant GM-29210 to A. F.

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