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Gating Movements of Colicin A and Colicin Ia Are Different

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Abstract. Both colicin A and colicin Ia belong to a subfamily of the bacterial colicins that act by forming a voltage-dependent channel in the inner membrane of target bacteria. Both colicin A and Ia open at positive and close at negative potential, but only colicin A exhibits distinctly biphasic turnoff kinetics, implying the existence of two open states. Previous work has shown that Colicin Ia gating is associated with the translocation of a region representing 4 of its alpha helices across the membrane. Also, if its C-terminal, channel-forming domain is detached from the other domains, its N-terminal alpha helix can now also cross the membrane, causing the conductance to drop by a factor of about 6. Colicin A gating also involves the translocation of an internal domain, but we find that its translocated domain is somewhat smaller than that of Ia. Furthermore, while its isolated C-terminal domain can also undergo a transition to a smaller conductance, the conductance change is only about 15%, and the transition does not involve the translocation of the N-terminal alpha helix. Trapping the N-terminus on the cis side prevents neither this small conductance transition nor the biphasic turn-off. So, while the gating of both channels involves large, currently inexplicable conformational changes, these motions are qualitatively different in the two proteins, which may be a reflection of the dissimilar kinetics of closing.

Key words: Colicin — Channel — Translocation — Voltage dependence — Gating

Introduction

The channel-forming colicins are a family of bactericidal proteins that kill sensitive strains of *E. coli* via the formation of voltage-dependent channels (Schein, Kagan & Finkelstein, 1978; Lakey & Slatin, 2001; Slatin & Kienker, 2003). They are soluble proteins that readily bind to lipid membranes and become, essentially, membrane channels, where they can be studied in isolation from other proteins. While the sequence homology of the channel-forming domains of the colicins is only partial (Parker et al., 1989), the structures of the five such domains that have been solved bear a remarkable similarity (Parker et al., 1989; Wiener et al., 1997; Elkins et al., 1997; Vetter et al., 1998; Hilsenbeck et al., 2004), which suggests that they all work by the same mechanism. There are differences among these channels in conductance and gating, but these differences appear minor next to their similarities. Wherever it has been examined, channel-forming colicins were found to make large, low conductance, pH-dependent pores that conduct both cations and anions (Schein et al., 1978; Raymond, Slatin & Finkelstein, 1985; Pressler et al., 1986; Lazdunski et al., 1988; Nogueira & Varanda, 1988; Bullock, 1992). Positive voltage opens the channels, and negative voltage closes them. However, colicin A has two kinetically distinct open states, whereas colicin Ia does not.

There is no plausible model extant of how these proteins form pores. However, persuasive evidence that colicin gating involves large transmembrane movement of the protein has been presented (Raymond et al., 1986; Slatin, Raymond & Finkelstein, 1986; Merrill & Cramer, 1990; Slatin et al., 1994; Qiu et al., 1996). In particular, colicin Ia has been shown

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Abbreviations: col A (Ia), colicin A (Ia); CT-A (Ia), the isolated C-terminal domain of colicin A (Ia); DTT, dithiothreitol; EPDP, N-[2-(biotinamido)ethyl]-3′-(2′-pyridylthio)propionamide; HPDP, N-[6-(biotinamido)hexyl]-3′-(2′-pyridylthio)propionamide; IPTG, isopropyl-B-D-thiogalactoside; SA, streptavidin; TCEP, Tris[2-carboxyethyl]phosphine hydrochloride; WT, wild type; βME, beta mercaptoethanol; V_o, the voltage of half-maximal current.

to transfer a region of itself corresponding to 4 alpha helices (out of the 10 alpha helices H1 to H10 that account for the bulk of its soluble structure) across the membrane as part of normal gating, and colicin A has been shown to be capable of translocating exogenous proteins inserted into the loop between H3 and H4 completely across the membrane (Slatin et al., 2001). Here we examine the translocating proclivities and gating of these two colicins in more detail with the aim of understanding the relationship between protein movements and channel gating.

Materials and Methods

DNA Constructs

Plasmid pLR1, which encodes the WT full-length colicin A, and its unique cysteine mutants (except K428C, N431C and K489C) have been previously described (Duché et al., 1994). K428C, N431C and K489C colicin A were constructed with the Stragene Quickchange mutagenesis kit. The single-cysteine mutants K422C, S451C A480C, F494C, E504C and A558C of the C-terminal fragment were obtained by site directed mutagenesis as described previously (Duché et al., 1994). A DNA fragment encoding the colicin A C-terminal domain (CT-A) was amplified by polymerase chain reaction (PCR) using appropriate 5′ and 3′ primers. Two different 5′ primers were used. The first one contained an *Nde*I site at its N-terminus (ttc gaa ttc cat atg gtt gcg gaa aaa gcc aaa cat g). The second one encoded a cysteine residue at position −1 and contained an *Nde*I site at its N-terminus (ttc gaa ttc cat atg tgc gtt gcg gaa aaa gcc aaa cat g). The 3′ primer contained the *Bam*HI site at its N-terminus (cc ggg ctc gag gga tcc tta atg tgc agg tcg gat tat). The resulting DNA fragments were cut by *Nde*I and *Bam*HI and ligated into pET-14b (Novagen) cut with the same enzymes. The resulting plasmids encode CT-A and (C^{-1}) CT-A.

Protein Expression

Whole colicin A and its cysteine mutants were expressed in *E. coli* cells by induction with 0.3 μg/ml mitomycin C. The protein was purified by ion exchange chromatography on a DEAE-CM Biogel column (Biorad) equilibrated at pH 6.8. Prior to chromatography, the cell lysate was treated with 1/5 volume 25% streptomycin sulfate to remove nucleic acids. CT-A and the point mutant C^{-1} CT-A were expressed in BL21(DE23) cells by induction with 1 mM IPTG and was purified on a Novogen His-Bind column.

Biotinylation

Colicin mutants bearing a unique cysteine were biotinylated with HPDP-biotin and EPDP-biotin as described previously (Qiu et al., 1996). Briefly, the protein, in pH 9 buffer, was reduced with 20 mM DTT for 2 h at room temperature, separated from the reducing agent on a Sephadex G-50 column in the absence of oxygen, and mixed with 1/20 volume of the 3 mg/ml biotinylating agent in dimethyl sulfoxide. The reaction mixture was dialyzed twice against a 4,000-fold volume of buffer (25 mM $NaBO_4$, 2 mM EDTA, 0.3 M NaCl, pH 9) and the degree of biotinylation was assayed by SDS polyacrylamide chromatography. In some cases, biotinylated colicin was further purified by affinity chromatography on a monomeric avidin column (Pierce). Only biotinylated colicin binds to the resin. It is eluted with 2 mM biotin and re-dialyzed.

Purification of Biotinylated C^{-1} CT-A Bound to Avidin

HPDP-biotin-C^{-1} CT-A (0.33 mg) that had been purified on a monomeric avidin column was incubated with 10 mg avidin (Calbiochem) in a volume of 0.25 ml (molar ratio avidin/colicin = 30; molar ratio biotin binding sites on avidin/biotin on colicin = 120). The mixture was run on a G-75 Superdex size-exclusion column (Pharmacia).

Membrane Experiments

Planar lipid bilayers were formed across a 100 micron hole in a Teflon partition by standard methods (Montal, 1974). The lipid (asolectin, from which sterol had been removed) was dissolved in pentane and layered on top of the buffers on either side of the partition. After the solvent had evaporated the membrane was formed by raising the levels of the buffers above the level of the hole. Electrophysiological measurements were made using a homemade voltage-clamp amplifier, and data was recorded on a (Micron) PC/(National Instruments) PCI-1200 I/O board and on a Physiograph (Narco Biosystems) recorder (Kienker, Jakes & Finkelstein, 2000).

Results

Autotranslocation in Whole Colicin A

Unique cysteine residues were engineered into colicin A at positions 422, 428, 431, 451, 480, 489, 494, 504 and 558 (Fig. 1). The proteins were expressed, purified and biotinylated as described. All of these biotinylated colicins formed channels in planar bilayers that were qualitatively like the wild type. The position of the biotinylated residue with respect to the membrane was probed with the water-soluble protein streptavidin, which binds nearly irreversibly to biotin. An illustrative experiment is shown in Fig. 2. Streptavidin added to the cis side (the side to which colicin was added) when channels of colA-480-biotin were closed caused a drastic reduction in the rate of channel opening (Fig. 2*A*). Thus, residue 480 is exposed to the cis solution in the closed state. Complementary experiments showed that residue 480 was not exposed to the trans side in the closed state. When the biotin is located further upstream, the effect of cis streptavidin is not to destroy the channels, but rather to shift the I/V curve to more positive values. In both cases, the experiment shows that the biotin had access to the cis compartment when the channel was closed. In those cases where binding to cis streptavidin still permitted channel opening, subsequent trans streptavidin had no effect, showing that the SA-bound channels were different from SA-free channels, and that their existence was not a consequence of un-binding from the streptavidin. A similar effect was observed in colicin Ia biotinylated at comparable positions (Qiu et al., 1996). In another experiment (Fig. 2*B*), streptavidin added to the trans

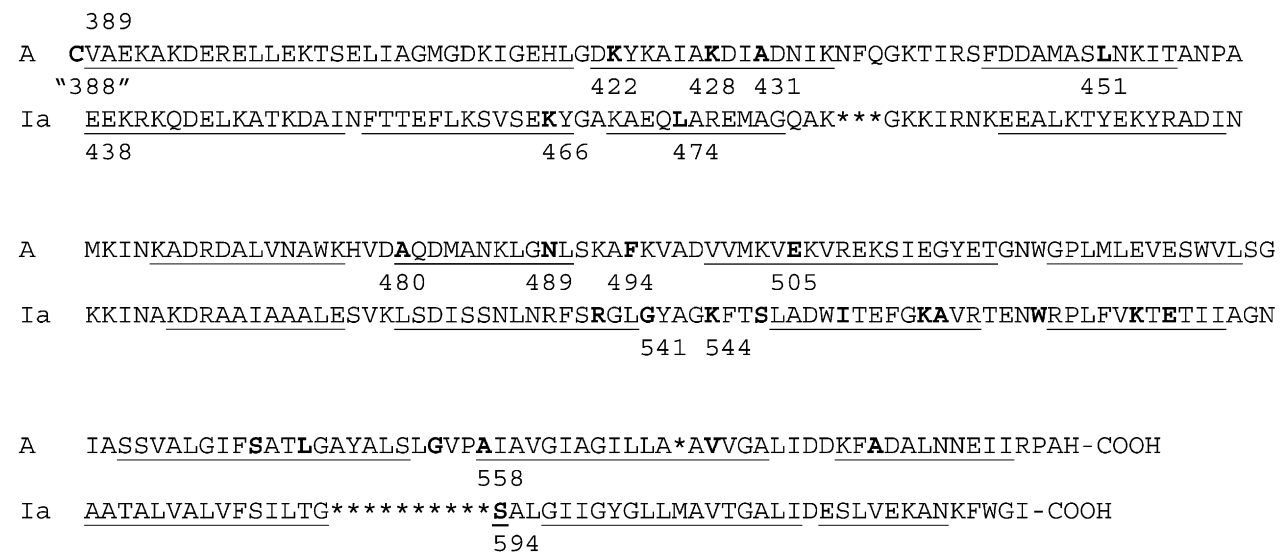


Fig. 1. Aligned sequences of the channel-forming domains of colicin A and Ia. The underlined residues denote the alpha helices in the crystal structure. Specific residues referred to in the text and in Table 1 are in bold type.

Table 1. Effect of Streptavidin on colicin A and colicin Ia biotin constructs

Colicin A biotin sites	388 N-H1	422 N-H2	428 M-H2	431 M-H2	451 M-H3	480 N-H5	489 C-H5	494 H5/H6	504 N-H6	558 N-H9
Cis effect	–	+	+	+	+	+	+	+	–	–
Trans effect	–	± pH 5/7	+	+	+	+	–	–	–	+

Colicin Ia biotin sites	466 C-H1	474 M-H2	541 loop H5/H6	544 loop H5/H6	594 loop H8/H9
Cis effect	+	+	+	+	+ (slow)
Trans effect	–	+	+	–	+

Membrane topology of open (+) and closed (–) colicin channels. *N*, *M* and *C* indicate the approximate location of the biotin (near the N-terminal end, middle or C-terminal end, respectively) in the particular helix. + and – indicate the presence or absence of an effect of streptavidin added to the indicated (Cis or trans) side of the membrane. Selected results with colicin Ia constructs are shown for comparison.

side when the channels of colA-451-biotin were open blocked turn-off, showing that residue 451 was exposed to the trans solution in the open state. The effect of streptavidin could be reversed by cleaving the disulfide bond linking the colicin to the biotin with reducing agents, such as βME and TCEP. Results for all the constructs are summarized in Table 1. Biotins placed at positions 428, 431, 451, and 480 are unambiguously detected on the trans side in the open state—trans streptavidin blocks channel closure, and the block can be relieved by adding a reducing agent to the trans solution. Exposure to cis streptavidin in the closed state affects all four of these constructs, consistent with the complete translocation of the region from (at least) 428 to 480 in association with gating. When the colicin is biotinylated at position 422, the second residue in H2, trans streptavidin has no effect at pH 7.0. At pH 5.0, however, trans streptavidin acts slowly to lock the channels in the open state. Long half times (~30 min) with the pH-insensitive reducing agent TCEP (25 mM) are required to reverse the effect. Biotins placed at positions 489, 494 and 504 do not evince a trans streptavidin effect. Cis streptavidin inhibits channel formation for 489 and 494, near the H5/6 loop, but not for 504, in H6.

The biotin at position 558 is exceptional among this group in that it is predicted to be in the middle of the hydrophobic segment, rather than in or near the translocated domain. This construct was designed to probe the location of the hydrophobic hairpin in the channel. Cis streptavidin had no effect on it, whereas trans streptavidin caused an increase in the on-rate. The trans streptavidin effect develops while the channels are closed, showing that the hydrophobic hairpin is inserted into the bilayer before the channel opens (Fig. 2*C*). This is consistent with results with colicin Ia biotinylated at the

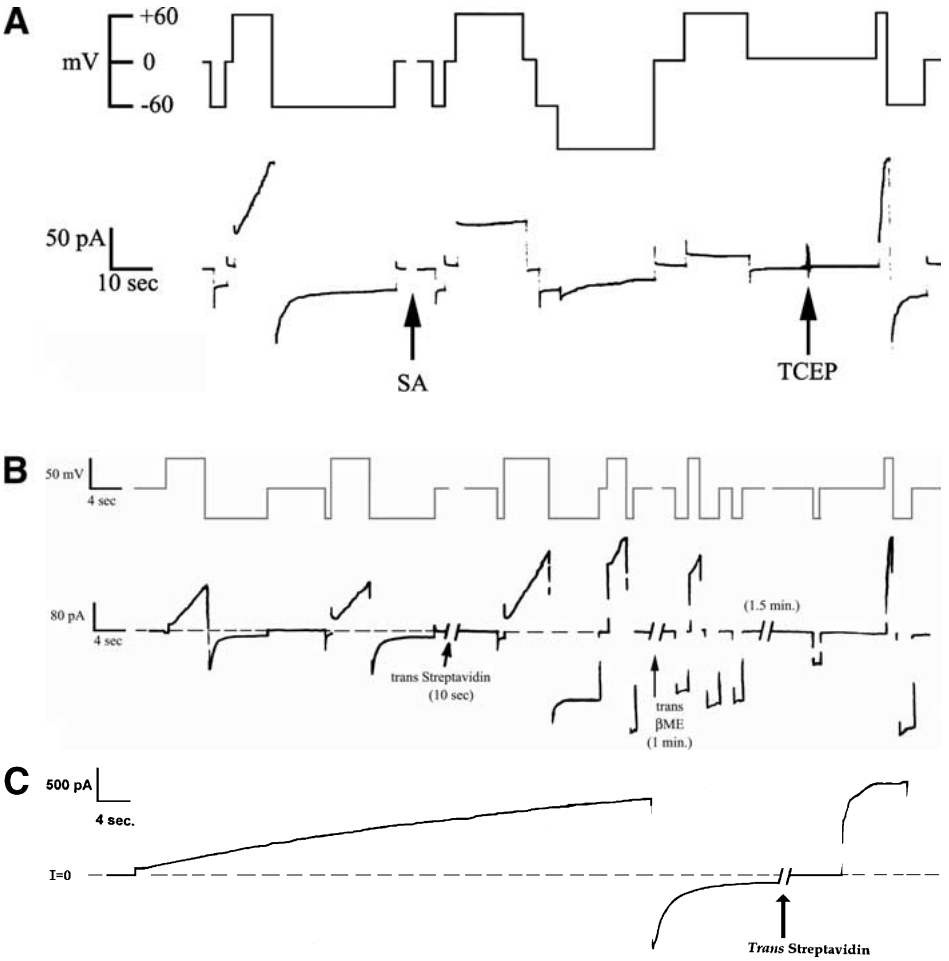


Fig. 2. Effect of streptavidin on biotinylated colicin A. (*A*) Colicin biotinylated in its translocated domain: ColA 480 cys-HPDP-biotin. Cis Streptavidin, 1 M KCl, pH 7.4; upper trace, voltage; lower trace, current. The record shows current continuously recorded from a single membrane with the voltage clamped as indicated. 20 μg of streptavidin was added to the cis compartment at the first arrow. Then, after a brief pulse to −60 mV (to verify that the conductance, which consists of channels in O_{slow} that were not closed by the previous −60 mV, had not changed) the voltage was stepped to +60 mV, which elicited no channel opening (compare to the +60 mV pulse before SA). After closing some of the channels in O_{slow} with a step to −120 mV, +60 mV again failed to open any channels. At the second arrow, 50 mM of the membrane-impermeant reducing agent TCEP was added to the cis compartment. Now, the final step to +60 mV turns on a large conductance. (*B*) Colicin biotinylated in its translocated domain: ColA 451 cys-HPDP-biotin. Trans streptavidin. At the beginning of the record, 2 sets of pulses to +50 and −50 mV established that the opening and closing rates were reproducible. After the addition of (20 μg) streptavidin to the trans side, channels opened as prior to SA addition, but now about 90% of the channels failed to close at −50 mV. The following +50 mV pulse opened more channels that were likewise locked open. The effect was reversed by adding the reducing agent βME to the trans compartment. When a new set of channels was subsequently opened with a short step to +50 mV, they too were locked open by the still-present trans SA, but then closed slowly at 0 mV as they were acted on by βME. (*C*) Colicin biotinylated in its hydrophobic domain: ColA 558-cys biotin. Trans streptavidin. Near the beginning of this current record, the voltage was stepped to +60 mV and held there while the conductance rose to about 1 nA. The polarity was then reversed, closing most of the channels. At the beginning of the 2 minute break in the record, 10 μg of streptavidin was added to the trans side while the channels remained closed. When the voltage was then stepped to +60 mV after the break, the on-rate was noticeably increased, compared to before streptavidin.

equivalent position (residue 594) (Kienker et al., 1997). 558-Biotin colicin A pre-incubated with streptavidin is inactive, as expected, so the lack of an effect of cis streptavidin on membrane-bound channels (*not shown*), even in the closed state, must be due to the inability of the streptavidin to gain access to the biotin (presumably because it is protected by the bilayer). This is not the case with colicin Ia, which can be washed off the membrane in the closed state.

Open States of the C-Terminal Fragment *vs.* the Whole Protein

The kinetics of closing of whole colicin A is biphasic, suggesting the presence of two open states,

which we call O_{fast} ($\tau = 1.2 \pm 0.6$ s at -60 mV, pH 7.5) and O_{slow} ($\tau > 10$ min.). In Fig. 3, the part of the conductance that remains after each fast turn-off at negative voltage represents channels in O_{slow} (Fig. 3*A*, whole colicin A; 3*B*, CT-A). The fraction of channels in O_{slow} increases with decreasing pH (*not shown*). The isolated C-terminal domain of colicin A (CT-A; residues 389 to 592) exhibits similar kinetic states, although the fraction of channels in O_{slow} is larger than that seen in the whole protein, especially at pH above 7. In order to ascertain the location of the N-terminus of CT-A in the open state (we cautiously assume that this locus is always on the cis side in wt colA) we introduced a cysteine (cys 388) on the upstream side of its first residue (along with a short 6-His tag to allow its purification). When this cysteine was biotinylated, we found no effect of either cis or trans streptavidin on any property of the channel. Furthermore, when the biotinylated peptide was preincubated with streptavidin, the resulting streptavidin/CT-A complex made normal channels that exhibited both open states (Fig. 3*C*). This rules out the explanation, suggested by analogy to colicin Ia, that O_{slow} is due to the translocation of H1 (*see* Discussion). In order to eliminate any possible contamination with unbound colicin, we purified the complex further by incubating the biotinylated CT-A (previously purified on a monomeric avidin column) with an excess of avidin, and then running the mixture on a sizing column (Superdex-75) to remove any possible contamination with unbound colicin. The colicin/avidin complex should have a MW of over 70 kDa (more, if more than 1 colicin were to bind to an avidin), compared to about 18 kDa for free CT-A.

Translocation of H1

In CT-Ia, when H1 is translocated, there is a transition to a smaller, 3-helix channel, manifested as a large drop in conductance (Kienker et al., 2000), shown in Fig. 4*A*. In the figure, two channels are seen to open, but only one undergoes the transition to the low-conductance state ($CT\text{-}Ia_{small}$) before the voltage is switched. Notice that the normal-sized channel closes almost immediately after the switch to -50 mV, while the $CT\text{-}Ia_{small}$ channel remains open more than two minutes. If CT-A were to gate like CT-Ia, we would expect to observe a similar phenomenon. We find that, under certain conditions, CT-A too makes a transition — but to a state whose conductance is only slightly smaller than the wt channel at 50 mV (Fig. 4*B*). The post-transition channel, $CT\text{-}A_{small}$, unlike CT-A, exhibits an almost 2-fold rectification between $+50$ mV and -50 mV, suggesting that it has a rather different structure, despite its similar conductance at $+50$ mV. As with colicin Ia, channels formed by the whole colicin A protein do not exhibit these conductance transitions (at least, under any of the several pH and voltage conditions we have used). CT-A channels tend to dwell longer than CT-Ia channels in the normal state before making the transition. In CT-Ia, the small, 3-helix channel has a much slower off-rate than the whole protein. CT-A channels that have undergone a transition are also slow to close, but we have not been able to determine if they differ kinetically from channels in the O_{slow} state, since some fraction of CT-A channels that have not undergone the conductance transition can themselves enter O_{slow}.

Does the transition seen with CT-A represent the translocation of H1 and the consequent loss of a transmembrane segment, as it does in CT-Ia? To test this, we looked at single channels of CT-A bound to avidin at their N-terminus, purified as above. We observed typical CT-A transitions (Fig. 4*C*). Thus, holding the N-terminus on the cis side does not prevent the transition in CT-A.

Discussion

The channels formed by the colicin family of proteins are an enigma, both structurally and mechanistically. On the structural side, there does not seem to be enough protein available to form a pore with the known properties of colicin channels. On the mechanistic side, gating seems to involve the transmembrane movement of a large portion of the colicin protein, but it is not at all clear how this movement relates to sensing the voltage or forming the pore. Given the strong homology among the solved water-soluble structures of the channel-forming domains of colicins A, Ia, E1, N and B, and the similarities observed among the channels they form, our working hypothesis is that colicin channels are fundamentally similar, both structurally and mechanistically. Here we examine this conjecture by comparing certain gating and translocation features of colicin A to the well-characterized colicin Ia, and we find significant differences that may shed light on how these proteins work.

The Hydrophobic Hairpin

Results with 558-biotin colicin A show that the hydrophobic hairpin is in a transmembrane orientation in both the open and closed state, as it is in colicin Ia (Kienker et al., 1997). Unlike colicin Ia, however, neither cis streptavidin nor perfusion of the cis chamber removes closed channels from the membrane. Evidently the hydrophobic hairpin of colicin A is more stably inserted into the bilayers than that of colicin Ia, perhaps because it is sig-

Fig. 3. Kinetic gating states of colicin A. (*A*) Wt colicin A, pH 6.1. Upper trace, voltage; lower trace, current; dashed line, zero current. During the first −60 mV pulse about 2/3 of the conductance turns off on a time scale of seconds. The remaining third closes imperceptibly slowly at this chart speed. A later pulse to −120 mV closes most of the channels in O_{slow}. The same behavior is repeated in a later series of pulses. Note the fast closing rate at $V = 0$. (*B*) CT-A, pH 5. A series of pulses to +50 and −50 mV shows that most of the conductance closes quickly at first, but that the number of channels in O_{slow} builds up with longer exposure to positive voltage, even though the maximal current in each +60 mV pulse is roughly the same. The final pulse, to −100 mV, closes the channels in O_{slow}. (Note: the current goes off scale briefly at the beginning of the final pulse.) (*C*) Ct-A bound to avidin, pH 5.0., 0.1 M KCl. The slow and fast open states are both present in this complex. In the first part of the record each of 3 pulses to +60 mV puts successively more channels into O_{slow}. A 5 second long step to −120 mV closes about half of them. After the break, during a series of short pulses to +60 and −60 mV, an increasing number of channels enter O_{slow}, while the maximal number of open channels remains constant.

nificantly larger (49 consecutive uncharged residues, *vs.* 40).

The Translocated Segment

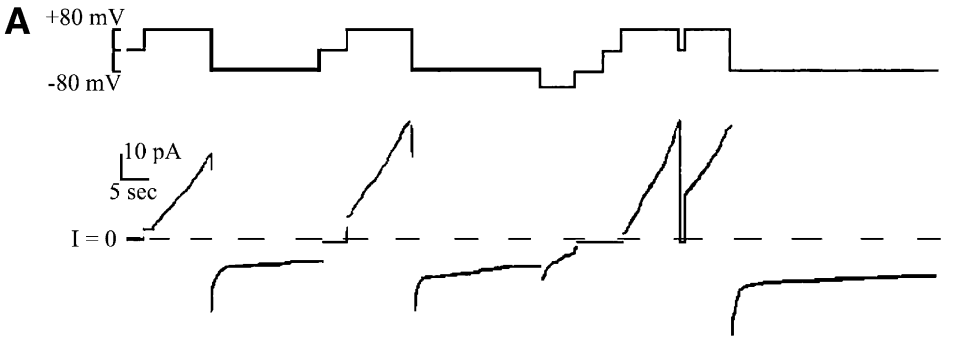
The gating of colicin Ia is associated with the displacement of the 2nd through the 5th alpha helix (of its 10-helix soluble structure) from the cis to the trans side of the membrane (Qiu et al., 1996). Colicin A also has been shown to posses a translocation function (Slatin et al., 2002). Our results here with site-directed biotinylation of whole colicin A show that it too has a translocated domain in its upstream half, but that it is not an exact homologue of that of colicin Ia. In this experiment, a unique biotinylated residue on the colicin serves as a potential attachment site for either avidin or streptavidin, both of which are large, soluble proteins. All of the biotinylated colicin A mutants used here formed normal channels in planar lipid bilayers, which allowed us to test the location of the biotin probe by looking for an effect of streptavidin added to one side of the membrane or the other, while monitoring the conductance. An effect on the channel in the open or closed state is interpreted as evidence that the biotinylated residue is on a particular side when the channel is in a particular state. The ability to block the effect with an excess of free biotin, and to reverse it by removing the biotin in situ (along with the attached streptavidin) with reducing agents (e.g., βME, DTT, TCEP) that cleave the disulfide bond connecting the biotin to the unique cysteine residue, allows us to control for any nonspecific effects (Slatin et al., 1994). It should be noted that the linker that attaches the biotin to the cysteine is about 15 Å long and so introduces some uncertainty into the position of the cysteine. The disulfide bond could be buried in the membrane 15 Å from the trans solution and still allow the biotin access to the aqueous phase. Since the biotin-binding pocket of streptavidin is buried in the interior of the protein, part of the length of the linker is necessary to reach the binding site. The minimal crystallographic distance is about 7 Å (Freitag et al., 1997), leaving 8 Å of free linker, but the effective maximal length of free linker may be less, since the experiment requires tight binding, and the binding affinity of tethered biotins is sensitive to spacer arm length (Green et al., 1971). The ability to reverse an effect of streptavidin with a reducing agent implies that the agent has access to the disulfide bond itself. However, the reducing agents used here are small molecules that should be able to enter the lumen of the open channel, and since we cannot rule out that reduction is via that route, we cannot interpret reversal to mean that the disulfide bond is exposed on the cis or trans face of the membrane.

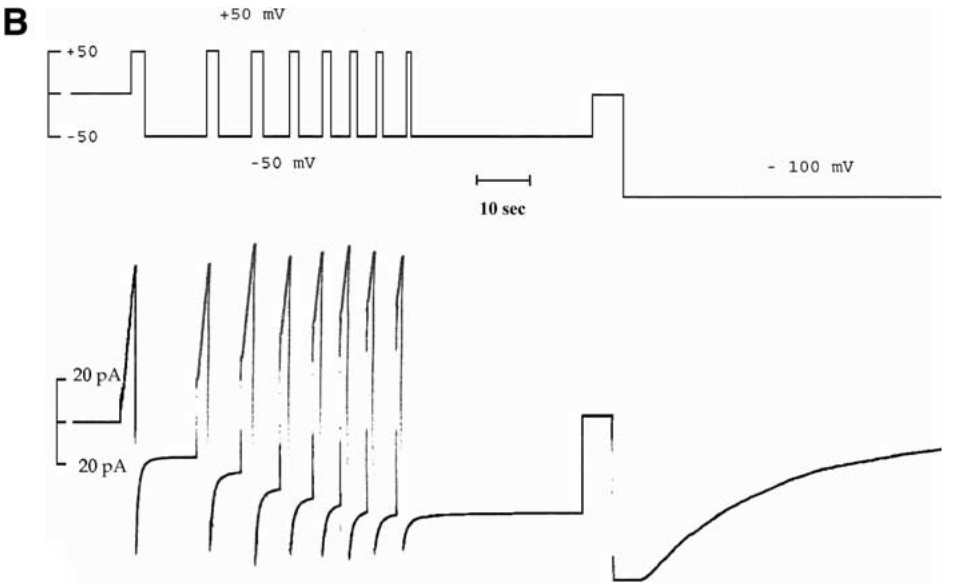
The C-Terminal Boundary of the Translocated Segment

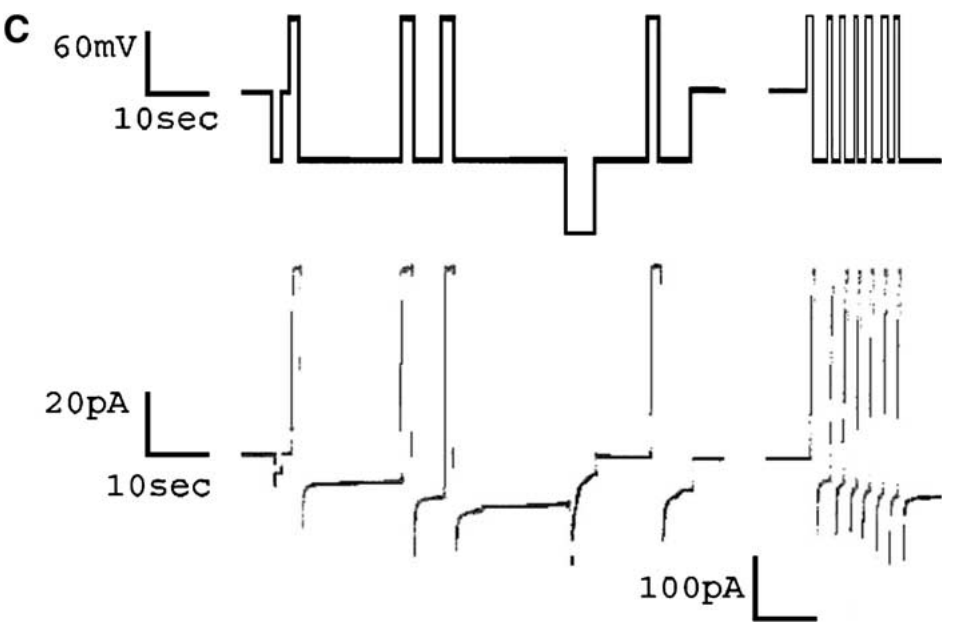
By these criteria residues 489, 494 and 504 of CT-A are not on the trans side in the open state. In the crystal structure these residues are in H5, the H5/6 interhelical loop, and H6, respectively. Since residue 480 of colicin A, the first residue of H5, gives a robust trans streptavidin effect, the C-terminal end of the translocated domain of colicin A lies within H5, and not at the H5/6 loop, as in colicin Ia. That this should be so might have been expected from the results of Nardi et al. (2001), which implied that H5 of colicin A plays a role in forming the channel, whereas in colicin Ia, H5 is inferred to be fully translated (Qiu et al., 1996).

The N-Terminal Boundary of the Translocated Segment

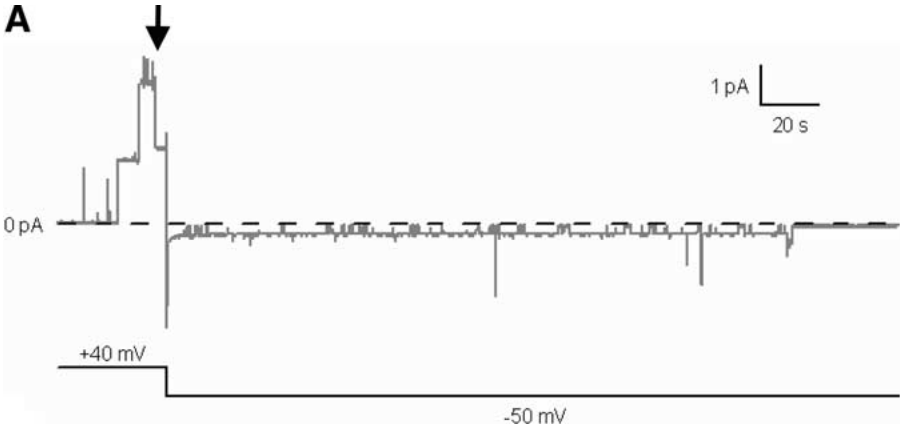
The boundary of the N-terminal end of the translocated segment in colicin Ia is between residue 466, in the last turn of H1, and residue 474, in H2, suggesting that the short H1/H2 loop forms a hinge between the transmembrane H1 segment and the translocated segment. In colicin A, residue 422, at the upstream end of H2, cannot be detected with streptavidin on the trans side at pH 7.0, although it can be at pH 5.0. At the low pH, however, the streptavidin effect is slow to develop and difficult to reverse, suggesting that residue 422 is sufficiently buried in the membrane so that the attached biotin is only marginally exposed to the

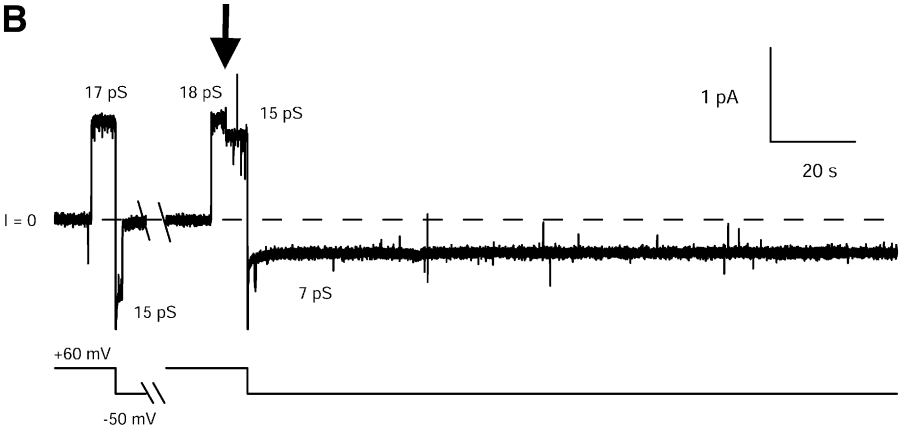






trans solution even at pH 5.0, and thus that residue 422 is on the borderline between trans accessibility and inaccessibility by our criterion. Residue 428-biotin, about half way along H2, is unambiguously on the trans side in the open state, thus locating the boundary between the upstream membrane-spanning segment and the translocated domain within H2. This is not incompatible with a strict structural homology to Ia, but it does rule out the crystal-structure H1/H2 loop as the boundary. Figure 5 compares schemati-





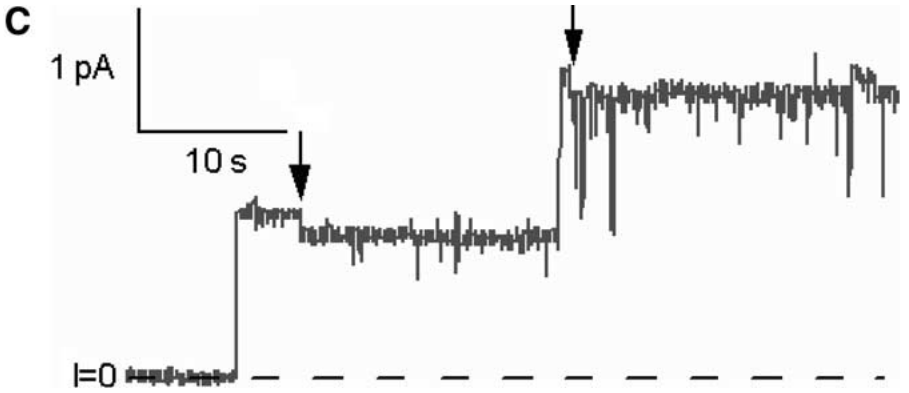


Fig. 4. Conductance transition of CT-Ia and CT-A. (*A*) CT-Ia. 1 M KCl, pH 6.2. Upper trace, current; lower trace, voltage. Two channels open near the beginning of this record, and one of them "closes" (at the arrow) to a low-conductance state. A few seconds later the voltage is switched to −50 mV, and the large channel, the one that had not undergone the transition, closes in about 1 second. The other, smaller channel remains open at −50 mV for more than 3 minutes. The dashed line represents zero current. (*B*) CT-A. 1 M KCl, pH 7.2. Upper trace, current; lower trace, voltage. In the first segment of the record, a single channel of 6-histidine CT-A opens at 60 mV and closes after about 2 s at −50 mV. In the second segment, a channel opens to a conductance of 19 pS and makes a transition (*arrow*) to 15 pS after 4 s at +60 mV. When the voltage is switched to −50 mV, the conductance is now 7 pS (this small channel is highly rectifying, whereas the large one is not) and it fails to close for the remainder of the record (in contrast to the pre-transition channel). (*C*) CT-A bound to avidin via a biotin at its N-terminal cysteine. 1 M KCl, pH 7.2, +70 mV. The biotinylated colicin had been purified on a monomeric avidin column, bound to an excess of avidin, and the bound complex was then purified on a Superdex column. During the record, two channels open and each undergoes a transition (*arrows*) to a smaller conductance state.

cally the topology of the open states of these two colicins as deduced from the whole-protein biotin experiments.

Kinetically Distinguishable Open States

The complex nature of colicin channel kinetics has frustrated efforts at quantitative analysis, but the gating of colicins A and Ia clearly differ qualitatively, in that colicin A has two kinetically distinct open states, whereas colicin Ia does not. A population of open colicin A channels clamped at −60 mV, say, closes with 2 disparate time constants. A fraction of the channels close with a time constant of about 1 second, while the remainder close in tens of minutes, at best (Fig. 3*A*). The time constant τ_{off} for channels in O_{fast} is not steeply voltage-dependent in this voltage range, so the channels close fast even at 0 mV, as seen in the figure. Channels in O_{slow} exhibit an increased τ_{off} and a shifted V_o, compared to O_{fast}, implying that there is both a heightened energy barrier and a shift in the free energy difference between it and the closed state. The observation that channels that are open briefly are usually in O_{fast} suggests that most or all channels do not enter O_{slow} directly from C. Thus, our simplified gating scheme is: Closed $\leftrightarrow$ $O_{fast} \leftrightarrow O_{slow}$.

Colicin A gating was analyzed by Collarini et al., who proposed that the protein exists in two states that differ markedly in their gating properties, and that the ratio of these states was dependent on pH (Collarini et al., 1987). In the form predominant at high pH ($colA_{basic}$), the channel behaves much like colicin Ia, with a V_o of +50 mV, so that all the channels are closed at, say, −50 mV, in the steady state. In the other, acidic, form, which dominates at low pH, V_o is −70 mV, so that a large fraction of the channels remains open at −50 mV. Furthermore, the isolated C-terminal domain (a bromelain fragment) was reported to gate like $colA_{acidic}$, even at basic pHs where the whole colicin is 100% in the basic form. That is, channels formed by the isolated fragment are mostly open at −50 mV. The two forms of the channel identified by Collarini et al. are probably closely related to the O_{fast} and O_{slow} states that we observe, but we do not presume that channels that are in O_{slow} and O_{fast} are equivalent to channels in $colA_{acidic}$ and $colA_{basic}$, respectively. $ColA_{acidic}$ and $colA_{basic}$ were identified from steady state measurements, whereas O_{fast} and O_{slow} are "kinetic" states, defined only by their τ_{off}. This lack of equivalence is demonstrated by, for example, the existence of O_{slow} in whole colicin A channels at high pH, and the existence of O_{fast} in the C-terminal fragment, even at low pH (Fig. 3*B*). In the figure, notice that after the first −50 mV pulse most of the conductance closes rapidly, but that the fraction in O_{slow} builds up with each subsequent pulse. We will speculate on the relationship between these various states below.

Wt colicin Ia, in contrast to wt colicin A, is reasonably well described with a single kinetic open state, which behaves much like O_{fast} of col A. The isolated C-terminal domain of colicin Ia (CT-Ia), however, has a second open state not seen in the channel formed by the whole protein (Kienker et al., 2000c). CT-Ia channels open initially into a normal open state (about 50 pS in 1 M KCl) and then undergo a transition to a much smaller conductance (about 7 pS in 1 M KCl). The first state is thought to be formed by 4 transmembrane segments (as is the channel formed by the whole colicin Ia protein) corresponding to approximately H1, H6/7, H8 and H9 of the crystal structure (Kienker et al., 2000). The small-conductance state (Ia_{small}) is thought to be formed when the upstream transmembrane segment (H1) flips over to the trans side, leaving a 3-segment channel. This small channel has a much slower τ_{off} than the normal channel, reminiscent of $colA_{deep}$. Presumably, the slower closing rate of Ia_{small} compared to Ia_{large} is due to the extra work needed to translocate H1 back into the membrane.

We wanted to see whether CT-A exhibited a state comparable to Ia_{small} of CT-Ia, both to test the functional homology of the two proteins and to search for an explanation for the deep open state of colicin A. Since deletion experiments had shown that CT behaves qualitatively normally without its first few helices (Baty et al., 1987; Nardi et al., 2001) we guessed that it would not mimic CT-Ia. We found that CT-A does indeed show a transition to a smaller conductance state, but that the difference in conductance was much less dramatic than in colicin Ia — a change of about 15% rather than 85% (at +70 mV). Nevertheless, like Ia_{small}, the "small" CT-A channel is slow to close. The association of the large conductance change in the colicin Ia transition and the coincident change from a 4- to 3-segment structure suggests that the smaller conductance is the direct result of a physically smaller lumen, but we cannot rule out the possibility that the conductance pathway of Ia_{small} and Ia_{large} are basically alike but that the presence of H1 in the membrane influences the conductance in some subtle way. In fact, the latter would probably be the inference one would make if the conductance drop were small, as in CT-A.

An important piece of evidence implicating the transport of H1 in the Ia transition was the observation that confining the N-terminal end of H1 to the cis side of the membrane by binding it to avidin blocked the transition (Kienker et al., 2000). When we now do the equivalent experiment with CT-A, we find that the transition is not blocked, showing that the transition in CT-A is not mechanistically the same as in CT-Ia. We cannot attribute it to a simple 4-segment to 3-segment alteration caused by removing H1 from the mem-

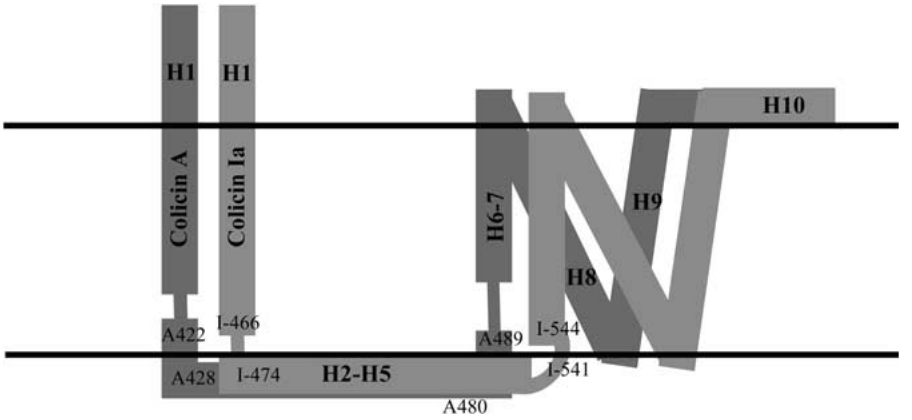


Fig. 5. Open-channel configuration of colicin A (*dark grey*) and Ia (*light grey*) as deduced from biotin/streptavidin experiments on the whole proteins. Some of the crystal structure helices are labeled with the prefix H. The loops between H1/H2 and H5/H6 of both proteins are drawn as narrower sections compared to the rest of the structure. The residues flanking the trans effect/no trans effect transitions are indicated with an *A* prefix for colicin A and an *I* prefix for colicin Ia. The colicin Ia residue numbers are the homologous colicin A residues (according to Parker et al., 1989), not the actual numbers of the Ia sequence. The bold horizontal lines represent the width of the membrane.

brane, since the upstream end of the molecule cannot translocate when bound to avidin. Might it yet be caused by the movement of some other transmembrane segment to the trans side? If so, the inference is that H1 does not initially form a transmembrane segment in the CT-A channel, but that something further downstream, a part of the protein whose homologue is normally on the trans side in the colicin Ia channel, forms the equivalent structure in CT-A, and that is what moves across the membrane to effect the transition. However, the translocation of such a segment would still leave the N-terminal on the cis side and would not change the number of transmembrane segments (at least in this avidin-bound configuration, and possibly without it). Such a model needs to be reconciled with the streptavidin experiments on whole colicin (Fig. 5), which consign H1 to the bilayer and most of H2-H5 to the trans side. Whole colicin A has not been observed to undergo a conductance transition, which may mean that it rapidly adopts a topology like the post-translation state of CT-A, with most of H2-H5 translocated and available for interaction with trans streptavidin. An explanation for the difference in behavior between whole colicin A and avidin-bound CT-A, however, remains obscure.

CT-Ia channels that have undergone the conductance transition have also undergone a transition to an altered gating state that closes slowly. This is easily understood in the context of the translocation of H1 — not only does its removal from the pore structure reduce the size of the channel, its presence on the trans side raises the energetic barrier to closure. Whole colicin A has a slow gating state but exhibits no transitions, suggesting that these two phenomena are unrelated. Furthermore, CT-A bound to avidin via a biotin at its N-terminus still exhibits both O_{slow} and O_{fast}, i.e., immobilizing H1 has no effect on either the conductance transition at the single-channel level or the slow gating state. We thus infer that the structural alteration that creates the O_{slow} in colicin A also occurs further downstream than H1.

In colicin Ia the transition to the deep open state that occurs when H1 translocates is prevented by the upstream domains of the whole protein. When these upstream domains are replaced by streptavidin, the channel continues to behave like the whole molecule. In colicin A, whatever conformational change accounts for the difference between O_{fast} and O_{slow} happens further downstream than H1, and is not prevented by tethering the upstream end of H1 to the cis side with avidin. Thus the ability of the upstream, non-channel-forming domains of colicin A to prevent the conductance transition is not due to their acting simply as a tether for the N-terminal end of the C-terminal domain; the upstream and C-terminal domains must interact in a more complex manner.

In this context it may be useful to compare the steady-state results (Collarini et al., 1987) with the kinetic experiments reported here. In the steady state, wild type colicin A channels at low pH, and CT-A channels at all pHs, are in state $colA_{acid}$, characterized by a large negative V_o, and are thus mostly open at, say, −50 mV; whereas we find that channels of either version of colicin A that have been open only briefly all close at −50 mV, i.e., they are in O_{fast}, which is more like $colA_{basic}$ (which has a positive V_o) than $colA_{acidic}$. Evidently the channels do not acquire their steady-state open conformation immediately, but must pass through an open state(s) that has a positive V_o along the way. The conformational changes that lead to the "final" open state may be translocation events, but, if so, they take place downstream of H1.

Perhaps O_{slow} is the result of one or more post-opening translocation events, and is equivalent to the open state observed for $colA_{acid}$ in the steady state, i.e., after long exposure to positive voltage.

Our results show that colicins A and Ia have important mechanistic differences, despite the striking structural similarity of their channel-forming domains. Colicin A apparently first opens into a shallow open state that does not make use of H1, and then undergoes-rearrangement(s) that stabilize the open state with little effect on conductance. Colicin Ia opens into a stable open state that does make use of H1 and that does not change drastically over time. These differences may be related to the different translocated domains of these two proteins.

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