

Identification of Channel-lining Amino Acid Residues in the Hydrophobic Segment of Colicin Ia

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Colicin Ia is a bactericidal protein of 626 amino acid residues that kills its target cell by forming a channel in the inner membrane; it can also form voltage-dependent channels in planar lipid bilayer membranes. The channel-forming activity resides in the carboxy-terminal domain of ~177 residues. In the crystal structure of the water-soluble conformation, this domain consists of a bundle of 10 α -helices, with eight mostly amphipathic helices surrounding a hydrophobic helical hairpin (helices H8-H9). We wish to know how this structure changes to form a channel in a lipid bilayer. Although there is evidence that the open channel has four transmembrane segments (H8, H9, and parts of H1 and H6-H7), their arrangement relative to the pore is largely unknown. Given the lack of a detailed structural model, it is imperative to better characterize the channel-lining protein segments. Here, we focus on a segment of 44 residues (573–616), which in the crystal structure comprises the H8-H9 hairpin and flanking regions. We mutated each of these residues to a unique cysteine, added the mutant colicins to the cis side of planar bilayers to form channels, and determined whether sulphydryl-specific methanethiosulfonate reagents could alter the conduction of ions through the open channel. We found a pattern of reactivity consistent with parts of H8 and H9 lining the channel as α -helices, albeit rather short ones for spanning a lipid bilayer (12 residues). The effects of the reactions on channel conductance and selectivity tend to be greater for residues near the amino terminus of H8 and the carboxy terminus of H9, with particularly large effects for G577C, T581C, and G609C, suggesting that these residues may occupy a relatively constricted region near the cis end of the channel.

INTRODUCTION

The colicins compose a family of proteins, made by *Escherichia coli* bacteria, which can kill competing strains of *E. coli* (Cascales et al., 2007). Each colicin has three functional domains: the central R domain binds to a receptor in the outer membrane; the amino-terminal T domain interacts with target cell proteins to move the carboxy-terminal domain (C domain) to the inner membrane; and the C domain carries the lethal activity. Colicins kill either by an enzymatic mechanism (Cascales et al., 2007) or by forming a voltage-dependent channel in the inner membrane (Cramer et al., 1995). All channel-forming colicins have, near the carboxy terminus, a distinctive hydrophobic segment of 31–49 consecutive uncharged amino acid residues, which stands out from the otherwise highly charged sequence. Several crystal structures have shown that this segment is roughly equivalent to the helix 8–helix 9 (H8-H9) hydrophobic hairpin, which is enclosed by the other eight helices of the C domain in its water-soluble conformation (Parker et al., 1989; Elkins et al., 1997; Wiener et al., 1997; Vetter et al., 1998; Hilsenbeck et al., 2004).

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Abbreviations used in this paper: C domain, carboxy-terminal domain; DT, diphtheria toxin; DTT, dithiothreitol; MMTS, methyl methanethiosulfonate; MTS, methanethiosulfonate; MTSES, sodium (2-sulfonatoethyl) MTS; MTSET, [2-(trimethylammonium) ethyl] MTS bromide; MTS-glucose, *N*-(β -D-glucopyranosyl)-*N'*-[(2-methanethiosulfonyl) ethyl] urea; V, reversal potential.

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We have determined some of the steps in channel formation by colicin Ia in planar lipid bilayer membranes, which most likely apply, at least qualitatively, to the other channel-forming colicins as well (Jakes et al., 1999). First, after colicin that was added to the aqueous solution bathing the cis side of the membrane binds to the membrane, the H8-H9 hairpin inserts into the membrane in a voltage-independent manner to form two transmembrane segments. Then, in response to a cis-positive voltage, an additional portion of the C domain inserts, so that parts of helices H1 and H6-H7 contribute the third and fourth transmembrane segments. Concomitantly, helices H2-H5 are translocated across the membrane to the opposite, trans side. This voltage-dependent insertion results in the opening of a conductive channel through the membrane.

Despite the delineation of the four transmembrane segments, the structure of the open colicin channel remains obscure. This is primarily due to the paradox of too little protein to make the channel, as discussed previously (Qiu et al., 1996). In brief, it appears that a single colicin monomer forms the channel with four transmembrane segments, and the pore diameter is at least 8–9 Å.

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Furthermore, under some conditions colicin Ia C domain can form a channel with only three transmembrane segments (Kienker et al., 2000), yet the channel diameter apparently remains large (unpublished data). It is even possible for large, folded peptides (up to 26 Å diameter), attached to the amino terminus of C domain, to be translocated across the membrane (Kienker et al., 2003). On its surface, this seems impossible. The most appealing proposal to resolve this paradox is that the membrane lipids may form a substantial part of the channel lining (Slatin, 1988; Sobko et al., 2006); however, the supporting evidence is indirect at best. Given these uncertainties, we cannot predict the locations of the four transmembrane protein segments *a priori*—whether all, some, or none line the pore—much less their proximity to one another, or the shape of the pore. There is evidence for colicin E1 channels, however, that mutations or chemical modifications that change the charge of certain residues in or near the hydrophobic segment (Shiver et al., 1988; Shirabe et al., 1989; Jakes et al., 1990; Song et al., 1991), as well as in another position (Abrams et al., 1991), produce changes in ionic selectivity, suggesting that the modified side chains may line the channel. Of course, there is always the concern that the mutations acted by perturbing the channel structure. Although the hydrophobic segment appears to insert as a helical hairpin (Song et al., 1991), no clear α -helical periodicity was established.

To clarify the situation, we now turn to the substituted cysteine accessibility method, which has been used to identify pore-lining residues in many channel proteins (Karlin and Akabas, 1998). In this method, a series of unique cysteine residues in a membrane channel is probed with a water-soluble, sulfhydryl-specific reagent. If the reaction influences the channel conductance or other properties, it indicates that the cysteine residue is exposed to the aqueous solution, rather than being buried in the lipid or the protein interior. In some cases, the pattern of reactivity has allowed the secondary structure of the channel-lining segments to be deduced. Here, we examine the 40 residues, 573–612, in the hydrophobic segment of colicin Ia (plus the four succeeding residues, 613–616, for reasons that shall be made clear). In the colicin Ia crystal structure, residues 573–575, 580–594, 597–612, and 614–616 constitute the end of helix H7, all of H8, all of H9, and the start of H10, respectively; the intervening residues 576–579, 595–596, and 613 belong to interhelical loops (Wiener et al., 1997). We shall attempt to determine to what extent these secondary structural elements are preserved in the open channel state.

MATERIALS AND METHODS

Construction and Purification of Mutant Colicin Proteins

All of the single cysteine mutants in colicin Ia were made using a plasmid, pKSJ101, which has the colicin Ia operon (the colicin

promoter region, colicin gene and immunity protein, from residue 711 to 3,347 of the Col Ia sequence; Mankovich et al., 1986), cloned between the unique EcoRI and BamHI sites of pUC19. Mutations were created using Agilent Technologies' QuikChange site-directed mutagenesis kit. All mutations were confirmed by DNA sequencing. The notation we use throughout for the mutant proteins, e.g., T573C, means that the threonine at residue 573 was mutated to cysteine.

Wild-type colicin E1 and the mutant C505A were purified from plasmids described previously (Jakes et al., 1990). Plasmid pKSJ331 was used for mutagenesis, using the QuikChange kit, to create colicin E1 mutant D473N. pKSJ331 was pUC19 with the colicin E1 operon cloned by PCR between the unique KpnI and BamHI sites of pUC19. The forward primer for the colicin operon included residues 5,015–5,035 of the ColE1 plasmid sequence, and the reverse primer included residues 499–479 (Chan et al., 1985). Thus, the *kil* or lysis gene of the E1 operon was not included, so the mitomycin C-induced cultures did not lyse.

Both colicin Ia and colicin E1 wild-type and mutant proteins were purified essentially as described previously (Jakes et al., 1990; Qiu et al., 1994), generally from 250- or 500-ml cultures. Dialyzed streptomycin-sulfate supernatants in 50 mM sodium borate, pH 9.0, 2 mM dithiothreitol (DTT), and 2 mM EDTA were purified on 1- or 5-ml pre-packed HiTrap CM FF columns (GE Healthcare). After washing the column extensively with the loading buffer, colicin was eluted with 0.3 M NaCl in the same buffer. All of the mutant proteins were expressed at wild-type levels and exhibited normal cytotoxic activity, as determined by spot-testing serial dilutions on sensitive indicator lawns. Yields were usually \sim 6 to 7 mg/250 ml culture. Stock solutions of proteins were stored frozen at -80°C . Aliquots thawed for use in the experiments were refrozen and kept at -20°C and were stable for months.

Planar Bilayer Experiments

Planar bilayers were formed from asolectin lipid using the Montal-Mueller technique, as described previously (Kienker et al., 2000). For most experiments, the bathing solution in the cis and trans compartments (1 ml each) was either 100 mM or 1 M KCl, with 5 mM CaCl₂, 1 mM EDTA, and 20 mM HEPES, pH 7.2. This pH was chosen empirically as one at which reactions of mutant colicin Ia channels with methanethiosulfonate (MTS) derivatives could be readily observed. In a few experiments, we used a gradient of 500 versus 50 mM TEACl, along with 5 mM CaCl₂, 1 mM EDTA, 5 mM HEPES, and TEAOH to pH 7.1. TEACl (Fluka) was used without purification, and TEAOH (Sigma-Aldrich) was purified as described previously (Slatin et al., 2008). The voltage-clamp recording system was as described previously (Kienker et al., 2000). The voltage is defined as the potential of the cis compartment, relative to that of the trans compartment. Ag/AgCl electrodes were connected to the bath compartments by 3 M KCl/3% agar bridges. DTT solutions were prepared daily from 1 M stock, which was stored at -20°C . For macroscopic experiments, the colicin stock solution (\sim 1 mg/ml) was mixed with an equal volume of 1% octylglucoside, plus DTT to 5 mM, and incubated for 5 min at room temperature. For single-channel experiments, the octylglucoside was frequently omitted. However, low-conductance channels were sometimes observed that resembled the channels formed by C domain fragments (Kienker et al., 2000). In this case, incubating with octylglucoside preferentially increased the number of normal-conductance channels, and single-channel experiments could be performed using high dilutions.

The addition of even low concentrations of DTT to the bath compartment sometimes destabilized the membrane, causing a noisy increase in conductance or membrane breakage. This problem could be solved by reforming the membrane. Consequently, as a preemptive measure, 5 μM DTT was typically added to one compartment before colicin addition. (This is comparable to the

amount of DTT added with the colicin.) In a few experiments, tris (2-carboxyethyl) phosphine (Thermo Fisher Scientific), another sulfhydryl-reducing agent (Getz et al., 1999), was used in place of DTT.

In specified experiments, colicin channels were held open with a positive voltage while the cis compartment was perfused with fresh bath solution to remove excess colicin. To avoid the possibility of cysteine oxidation (due to interaction between two colicin molecules, for instance), 5 μ M DTT was typically included in the perfusing solution.

The MTS reagents used were [2-(trimethylammonium) ethyl] MTS bromide (MTSET), sodium (2-sulfonatoethyl) MTS (MTSES; Toronto Research Chemicals and Biotium), methyl MTS (MMTS; Sigma-Aldrich), and N-(β -D-glucopyranosyl)-N'-(2-methanethiosulfonyl) urea (MTS-glucose; Toronto Research Chemicals). The chemical formulas for the MTS reagents have the form $\text{CH}_3(\text{SO}_2)\text{S}-\text{R}$, where R is $\text{CH}_2\text{CH}_2\text{N}(\text{CH}_3)_3^+$ for MTSET, $\text{CH}_2\text{CH}_2\text{SO}_3^-$ for MTSES, CH_3 for MMTS, and $\text{CH}_2\text{CH}_2\text{NH}(\text{CO})\text{NH}(\text{C}_6\text{H}_{11}\text{O}_6)$ for MTS-glucose. The product of reaction with Cys-SH is Cys-S-S-R. Stock solutions of 20 mg/ml were stored at -20°C . Typically, the final concentrations in the bath solution were 200 $\mu\text{g}/\text{ml}$ for MTSET and MMTS and 600 $\mu\text{g}/\text{ml}$ for MTSES and MTS-glucose (i.e., all $\sim 1\text{--}2\text{ mM}$).

$\text{Hg}(\text{C}_6\text{F}_5)_2$ (Andersen et al., 1976) was used as a Cl^- carrier to determine the Cl^- potential in experiments with a TEACl gradient.

The program Acquire (5.0.1; Bruxton Corp.) was used for data acquisition with a PCI-1200 A/D board (National Instruments); the sampling interval was 6 ms, but only every 10th point was plotted in the figures. Igor Pro (5.0.5.7; WaveMetrics, Inc.) was used for data analysis.

RESULTS

The aim of this paper is to identify the residues in helices 8 and 9 that line the channel lumen. To this end, only the single-channel MTS experiments are relevant. To rationally proceed with these, however, we first screened the residues in helices 8 and 9 with macroscopic MTS experiments. Any residues not giving a macroscopic effect were excluded from single-channel scrutiny, and

even some of those giving a macroscopic effect were also excluded for reasons given below. In a sense, we could have presented just the single-channel data, but we feel it is useful to include the macroscopic data so that the reader can fully appreciate the issues involved in determining the channel-lining residues.

Screening for Macroscopic Effects of MTS Reagents

Each of the 40 colicin Ia mutants with a single cysteine in the hydrophobic segment, 573C through 612C, generated a voltage-dependent macroscopic conductance in planar bilayers, similar to that of wild-type colicin Ia (Jakes et al., 1999). We wanted to test each of these mutants for sensitivity to MTS reagents. Colicin Ia channels are moderately selective for cations over anions at pH 7.2, so we chose the cationic MTSET for the initial series of experiments, in 100 mM KCl buffer. The typical experimental protocol was to open channels quickly at +70 mV, step to +50 mV, at which the opening rate was slow, wait a couple of minutes to establish a steady trend, and then add MTSET to one of the bath compartments. Fig. 1 shows a representative experiment with N578C, in which trans MTSET caused a decrease in conductance of $\sim 25\%$. The effects of trans and cis MTSET on all the mutant channels are summarized in Fig. 2. Not surprisingly, wild-type colicin Ia, which has no cysteine residues, showed no effect of cis or trans MTSET.

Before proceeding further, we need to make a few observations about the likely causes of these macroscopic effects: namely, changes in single-channel conductance versus effects on channel gating. In the simplest case, we expect an effect on the single-channel conductance to satisfy the following conditions. (1) The change in current should have a quasi-exponential time course (after a stirring delay), corresponding to the rate of

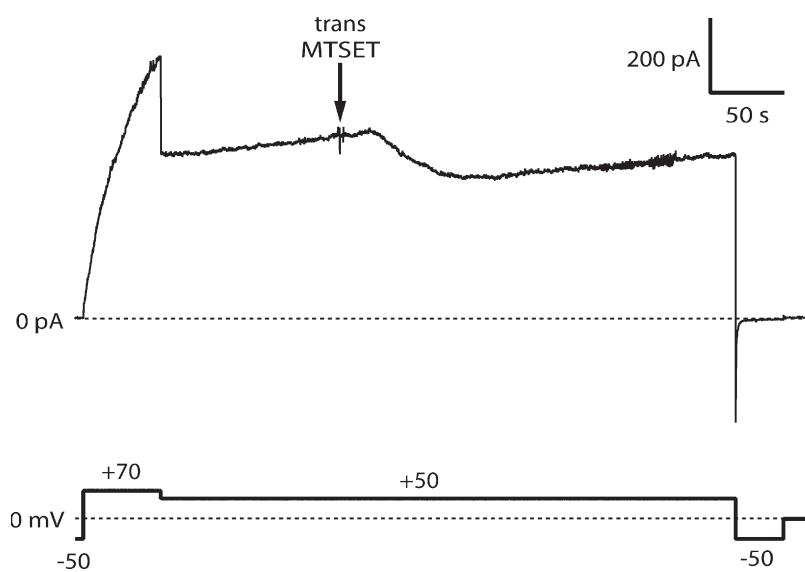


Figure 1. Effect of trans MTSET on the macroscopic current through colicin Ia mutant N578C channels. The top trace shows the membrane current and the bottom trace shows the voltage, each as a function of time. Before the start of the record, DTT was added to the trans compartment to a final concentration of 5 μM and 1.0 μg N578C (along with 4.5 μg octylglucoside and DTT to 5 μM) was added to the cis compartment. We quickly opened on the order of 1,000 channels by stepping the membrane potential to +70 mV, and then switched it to +50 mV to establish a slower channel-opening rate. At the arrow, 200 μg MTSET was added to the trans compartment. This caused a decrease in current of $\sim 25\%$, demonstrating that residue N578C was accessible for reaction. Finally, we confirmed that the channels closed normally when the membrane potential was switched to -50 mV . The solution on both sides of the membrane was 100 mM KCl, 5 mM CaCl_2 , 1 mM EDTA, 20 mM HEPES, pH 7.2. (The membrane broke after colicin addition and was reformed before the start of the record; similar results were obtained with membranes that had not broken.)

reaction. With two exceptions (G593C and S594C, see below), all the responsive mutants satisfied this condition. (2) MTSET should cause a decrease in conductance if it acts by either a direct electrostatic effect or a steric effect on K^+ permeation. (3) Cis and trans MTSET should have similar effects if the cysteine residue is accessible from both sides. All three conditions were satisfied by G577C, N578C, T581C, A585C, I598C, G602C, M605C, G609C, and possibly L586C (which had a very small trans MTSET effect); these are likely candidates for single-channel conductance effects of MTSET. If we relax condition 3 to allow for reaction from only one side, we can add A582C and F588C on the trans side and T573C, I574C, A606C, T608C, and I612C on the cis side. (We caution the reader on the interpretation of the cis side-only effects: MTSET may have reacted with colicin in the cis solution, thereby preventing new channels from forming, or it may have reacted in a closed state in which the hydrophobic hairpin is accessible to the cis side of the membrane [see, for example, Kienker et al., 1997].) Mutants satisfying neither condition 2 nor 3 were S589C, G593C, S594C, G597C, and

Y601C. For these, trans MTSET caused a conductance increase, whereas cis MTSET either caused a decrease or had no effect. At this point, a change in channel gating seems the most likely explanation for these MTSET effects. (For instance, reaction with trans MTSET may trap the hydrophobic hairpin in a transmembrane orientation, whereas cis MTSET may trap it near the cis side of the membrane, thus favoring an open or closed channel state, respectively.) This applies in particular to G593C and S594C, which did not even satisfy condition 1; the main effect of trans MTSET was, apparently, to produce a large, steady increase in the channel opening rate. As we shall see, these preliminary assignments are not perfect, but they make a good starting point. The remaining 19 mutants showed no effect of MTSET from either side.

In preparation for single-channel experiments, selected mutants were tested with MTSET in 1 M KCl buffer (Table I). The effects were generally comparable to the effects in 100 mM KCl buffer, although for several mutants (T573C, G577C, N578C, A582C, and A585C) they were smaller in magnitude. This is to be expected,

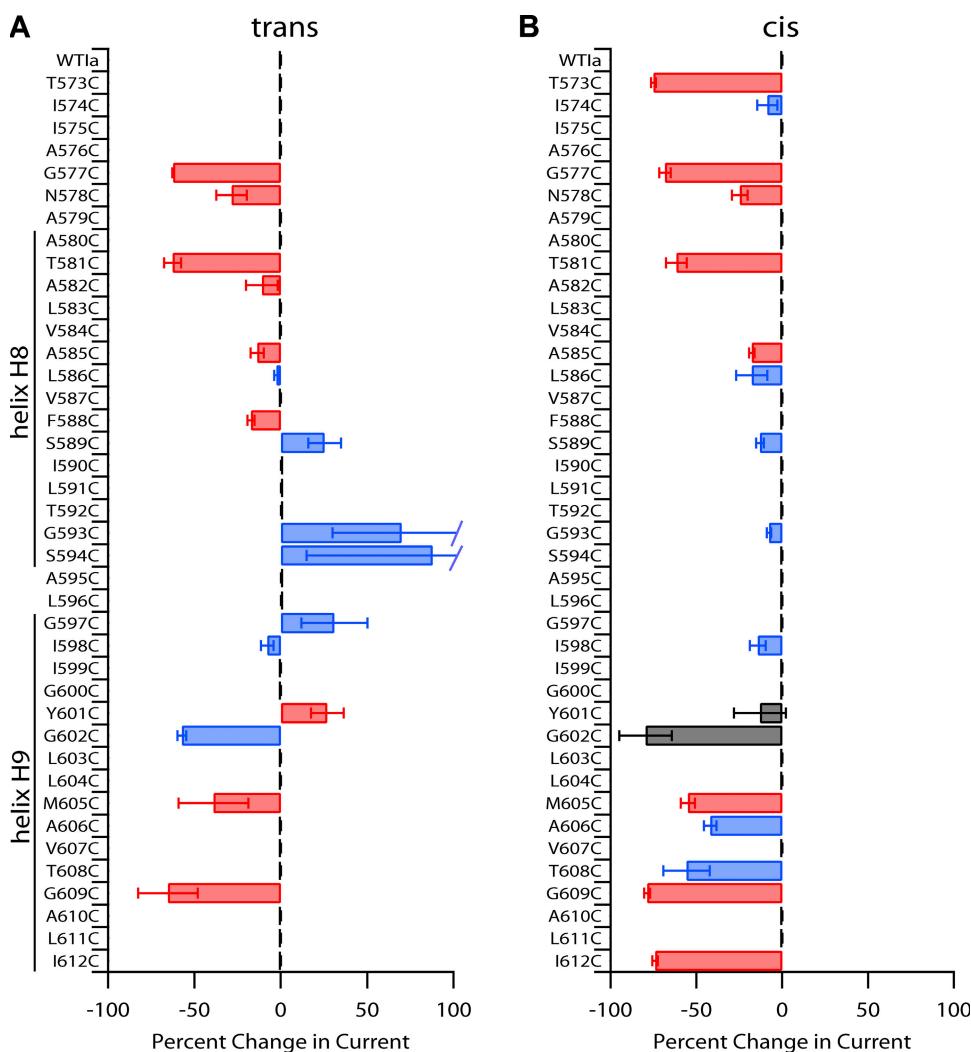


Figure 2. Summary of MTSET effects on the macroscopic current through colicin Ia cysteine mutant channels and wild-type. (A) Trans MTSET. (B) Cis MTSET. For mutants showing a response to MTSET, each bar represents the percent change in current (mean \pm SD). At least three experiments were performed for each condition. For mutants G593C and S594C with trans MTSET, the error bars were truncated so that the data for the other mutants could be displayed more clearly. The bars are color-coded as follows: red, has a single-channel MTS effect; blue, no MTS effect after cis perfusion. Of the remaining (gray) bars, the effect of cis MTSET on Y601C was not observed consistently (as reflected in the large standard deviation) and hence was not considered to be a real effect. Cis MTSET had a very slow effect on G602C, as described in the text, but an effect on single channels was not demonstrated. The lines on the left indicate the limits of helices H8 and H9, as defined by the crystal structure. Solutions were as in Fig. 1.

due to the increased screening of the appended positive charge by the electrolyte. For several other mutants (T581C, F588C, M605C, G609C, and I612C), the effects in 0.1 and 1 M KCl were similar in magnitude, and for Y601C the effect appeared to be somewhat larger in 1 M KCl.

These mutants were also tested with MTSES; some showed an increase in conductance, whereas others showed a decrease (Table I). We expect a conductance increase from the electrostatic effect of the appended anionic group on K^+ conduction; a decrease suggests that steric effects dominate. Thus, the decreases seen with G577C, T581C, and G609C indicate that their cysteine residues are situated in a narrower part of the pore than the other residues.

The issue of the relative pore diameter was further pursued by using the smaller, uncharged reagent MMTS. (Unlike MTSET and MTSES, MMTS is membrane permeant, but that will not affect our conclusions.) The effects of MMTS on all of these mutants are summarized in Table I. The larger conductance decreases observed for G577C and G609C support the notion that they are in a more constricted region of the pore. Significant decreases were also seen for T581C, M605C, N578C, and A585C, ordered from greatest to least effect, as well as an increase for Y601C. We also determined the effects of trans MTS-glucose, a larger, uncharged reagent, on G577C, T581C, and G609C (Table I). In each case, MTS-glucose caused a large decrease in conductance, exceeding the effect of MMTS, as expected.

MTS Effects on Single-Channel Conductance

Colicin Ia channels have a rather broad distribution of conductance values (see, e.g., Krasilnikov et al., 1998).

All of the mutants listed in Table I had, before reaction, a more or less normal single-channel conductance in 1 M KCl buffer, typically in the range of 40–60 pS at +50 mV and slightly larger at higher voltages. For example, we measured the G609C single-channel conductance (from the initial series of channel openings at the beginning of macroscopic experiments) as 52 ± 6 pS ($n = 33$ channels in eight experiments). Of the other channels that we inspected, I574C had the largest conductance, ~ 80 pS at +50 mV.

The mutant channels that displayed macroscopic MTSET effects were examined at the single-channel level. 11 of these mutants responded to MTSET, MTSES, or MMTS with a step change in the single-channel conductance (Table I; red bars in Fig. 2). Representative examples are shown of the reactions of F588C channels with MTSET (Fig. 3 A), A585C with MTSES (Fig. 3 B), and G609C with MMTS (Fig. 3 C). The single-channel effects were more or less in line with the macroscopic effects for these mutants, although the single-channel effects were typically somewhat larger in magnitude. Consistent with our a priori expectations, MTSET caused a conductance decrease for almost all of the channels listed in Table I. The exception was Y601C, which, rather surprisingly, showed an increase in single-channel conductance, comparable to the increase seen in macroscopic experiments. This indicates that reaction with MTSET caused a change in the channel conformation. The increases and decreases induced by MTSES matched the macroscopic results. The mutants showing the largest macroscopic effects of MMTS gave quite similar decreases in single-channel conductance. Although we have not made an extensive study of the issue, all the reactions were manifested as single-step conductance

TABLE I
MTS Effects on the Conductance of Colicin Ia Cysteine-mutant Channels

| Mutant | Percent Conductance Change | | | | | | | |
|--------|----------------------------|-------------------|--------------------|------------------|--------------------|---------------------|---------------------|---|
| | MTSET | | MTSES | | MMTS | | MTS-glucose | |
| | Macroscopic | Single | Macroscopic | Single | Macroscopic | Single | Macroscopic | |
| T573C | -47 ± 4 (3) | -58 ± 2 (6) | $+2 \pm 2$ (2) | — | NE (2) | — | — | — |
| G577C | -48 ± 6 (2) | -82 ± 1 (3) | -29 ± 8 (3) | -45 ± 6 (26) | -29 ± 6 (3) | -33.7 ± 0.5 (3) | -74 ± 6 (2) | — |
| N578C | -8 ± 4 (2) | -56 ± 10 (4)* | $+5 \pm 2$ (3)* | — | -6.5 ± 0.5 (2) | — | — | — |
| T581C | -62 ± 2 (3) | -75 ± 1 (5) | -20 ± 2 (2) | — | -16 ± 7 (4) | -12 ± 3 (4) | -28 ± 10 (3) | — |
| A582C | -5.5 ± 0.5 (2) | -25 ± 5 (3) | $+4 \pm 4$ (3) | — | NE (3) | — | — | — |
| A585C | -7 ± 2 (3) | -11 ± 5 (10) | $+23 \pm 2$ (3) | $+31 \pm 6$ (28) | -4 ± 1 (4) | — | — | — |
| F588C | -23 ± 2 (6) | -25 ± 5 (9) | $+12 \pm 25$ (3) | — | NE (4) | — | — | — |
| Y601C | $+47 \pm 3$ (3) | $+37 \pm 5$ (7) | $+92 \pm 2$ (2) | $+76 \pm 3$ (4) | $+16 \pm 2$ (3) | — | — | — |
| M605C | -38 ± 13 (2) | -40 ± 6 (7) | $+15 \pm 2$ (3) | — | -12 ± 7 (4) | — | — | — |
| G609C | -63 ± 3 (4) | -74 ± 6 (4) | -31 ± 6 (2) | -59 ± 2 (8) | -32 ± 7 (4) | -35 ± 4 (3) | -49.5 ± 0.5 (2) | — |
| I612C | -68 ± 10 (3) | -73 ± 10 (6) | $+9.5 \pm 0.5$ (2) | — | NE (2) | — | — | — |

Percent change in macroscopic and single-channel conductance of colicin Ia cysteine mutants due to reaction with MTS compounds. All results were obtained in 1 M KCl, pH 7.2 buffer, except those marked with an asterisk, which were in 100 mM KCl buffer. MTS reagents were added to the cis compartment for T573C and I612C and to the trans compartment for all of the other mutants. The percent change in conductance is given as the mean \pm standard deviation, with n (the number of macroscopic experiments or the number of single-channel reaction events) in parentheses. NE means no effect. The holding potential during MTS addition was in the range of 20–80 mV.

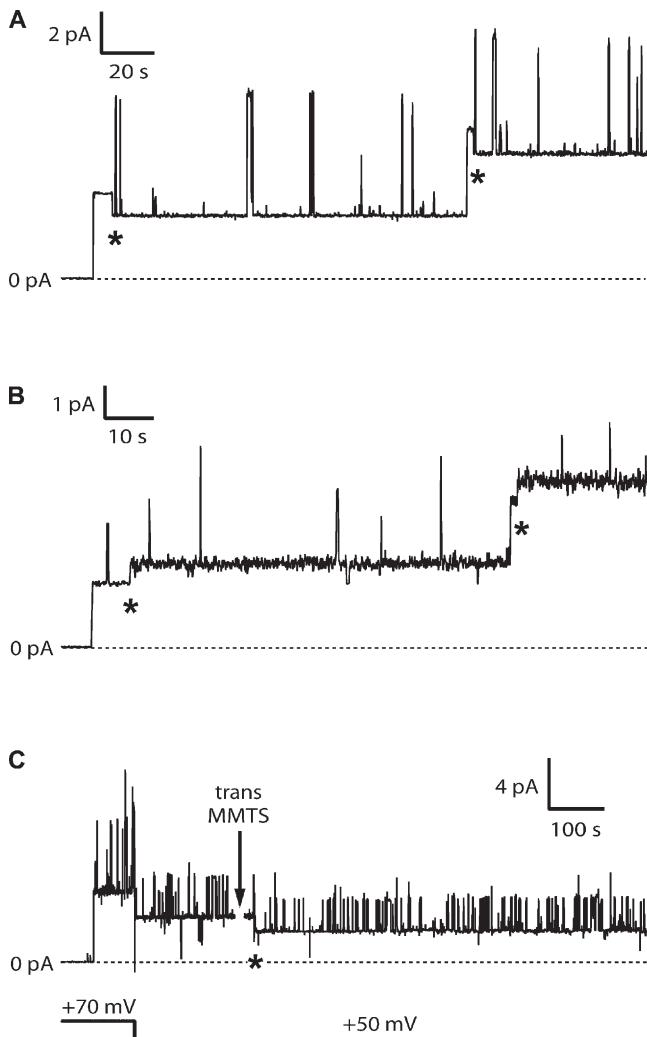


Figure 3. Effects of various MTS reagents on the single-channel conductance of colicin Ia cysteine mutants. Each MTS reaction event is highlighted by an asterisk. (A) Effects of trans MTSET on F588C. Before the start of the record, F588C was added to the cis compartment, and a total of 300 μ g MTSET was added to the trans compartment on two occasions, with the latter addition occurring 16 min before the start of the record. The record shows single-channel currents at a constant membrane potential of +70 mV. First, a 59-pS channel opened. After 7 s, the conductance dropped to 43 pS (a 27% decrease), representing the channel's reaction with MTSET. Similarly, a second 60-pS channel opened and then dropped to 43 pS (a 28% decrease) 2 s after opening. In this analysis, we neglect the brief flickers to a higher conductance state (noted by Kienker et al., 2003) that are evident in this record, as well as in B and C. (B) Effects of trans MTSES on A585C. This mutant was particularly reactive, so we used less MTSES than usual, allowing the reactions to be observed on a convenient time scale. A585C was added to the cis compartment and, 15 min before the start of the record, 20 μ g MTSES was added to the trans compartment. The record shows single-channel currents at +40 mV. First, a 48-pS channel opened and, 8 s later, increased in conductance to 64 pS, indicating reaction with MTSES. There was also a notable increase in current noise upon reaction. A second channel likewise showed a conductance increase from 47 to 62 pS, occurring 1 s after its opening. (The increases were 33 and 32%, respectively.) (C) Effect of trans MMTS on G609C. Before the start of the record, G609C was added to the cis compartment.

changes, suggesting that there was only one reaction, and hence only one cysteine residue, per channel; i.e., the channel is a colicin monomer.

Elimination of MTSET Effects on Channel Gating

In contrast to the 11 mutants in Table I that displayed a single-channel conductance change upon reaction, several other mutants that showed macroscopic MTSET effects did not readily reveal an effect on single-channel conductance. These included L586C, S589C, G593C, and G602C. In addition, the response to MTSET of several mutants was inconsistent with a simple electrostatic or steric mechanism of action. This suggested that the macroscopic effects might be due instead to changes in the rate of channels opening or closing. (In experiments with mutant G593C at the single-channel level, we could see that trans MTSET indeed caused a marked increase in the channel opening rate.) We sought to minimize these effects on channel gating by means of the following experimental modifications. (1) We perfused the cis compartment with fresh bath solution to remove excess colicin, as described in Materials and methods. (2) We held the membrane potential at larger values (60–80 mV) to ensure that open channels would stay open. (3) If the effect persisted in 100 mM KCl buffer, we also tried 1 M KCl buffer, with the idea that greater screening of the charge on MTSET might reduce its effect. This was justified because we ultimately wanted to use the 1 M buffer for single-channel experiments. For several of these mutants, cis perfusion at +50 mV led to a slow decrease in conductance, confirming the need to use larger voltages to keep the channels open. When the following mutants were tested using this protocol, the MTSET effects were eliminated: in 100 mM KCl buffer, L586C (cis and trans), S589C (cis),

The record begins with a channel opening at +70 mV. The membrane potential was then lowered to +50 mV, at which the single-channel conductance was 70 pS. (This conductance is a little higher than average, but not shockingly so.) Next, at the arrow, 200 μ g MMTS was added to the trans compartment. After a delay of 28 s, the conductance dropped to 48 pS (a 31% decrease), reflecting reaction of G609C with MMTS. For this record, we were able to analyze the brief flickers to a higher conductance state, whose conductance also decreased upon reaction with MMTS, from 133 to 99 pS (a 26% decrease). (The conductance states were identified clearly by an all-points histogram analysis.) For cosmetic reasons, we suppressed the current trace at the time of MMTS addition, but the passage of time is represented accurately despite this break. The voltage trace applies only to C. For all three panels, the solution on both sides of the membrane was as in Fig. 1, except the KCl concentration was 1 M. The dashed line in each panel indicates the zero current level. The amounts of mutant colicin, octylglucoside, and DTT added to the cis compartment and DTT added to the trans compartment before the start of each record were, respectively, 24 ng, 0, 21 μ M, and 50 μ M in A; 2.1 ng, 10 μ g, 10 μ M, and 5 μ M in B; and 3.4 ng, 0, 6 μ M, and 5 μ M in C.

G593C (cis), S594C (trans), G597C (trans), and A606C (cis); and in 1 M KCl buffer, I574C (cis), S589C (trans), G593C (trans), I598C (cis and trans), G602C (trans), and T608C (cis) (Fig. 2, blue bars). Even with this protocol, mutant G602C showed a large, extremely slow decrease ($t_{1/2} \sim 400$ s) in response to cis MTSET at potentials up to +80 mV. Because this effect was so slow, we did not pursue it further in single-channel experiments. As positive controls, F588C channels gave their usual trans MTSET effect and M605C channels their usual cis MTSET effect with this protocol in 1 M KCl buffer, and T573C, A585C, and I612C showed their cis MTSET effects when they were tested in 100 mM KCl buffer.

In general, the effects of cis MTSET that we have now eliminated provide no information about the structure of the open channel because they could have been due to reaction with colicin in the cis solution. On the other hand, the effects of trans MTSET on S589C, G593C, S594C, and G597C are more informative. Although these residues are not located where they can influence the single-channel conductance, they are accessible to trans MTSET in some membrane-inserted state of the colicin. Presumably, trans MTSET reaction with closed channels was able to trap the H8-H9 hairpin in a trans-membrane orientation, thereby shifting the equilibrium toward channel opening (Kienker et al., 1997); cis perfusion eliminated this effect by removing the pool of closed channels.

MTS Effects on Ionic Selectivity

The effect of MTSET on the colicin Ia channel's selectivity for K^+ versus Cl^- was assayed by measuring the reversal potential (V_r) with a KCl gradient across the membrane of 100 mM cis/1 M trans, both sides at pH 7.2. An example with G577C is depicted in Fig. 4. The V_r changed from +30 mV before MTSET addition to

-18 mV after, reflecting a large change in selectivity from favoring cation permeation to preferring anions. The results of a more extensive series of measurements are shown in Table II. Although many of the reactive positions showed only minor changes in selectivity upon reaction with MTSET, quite large effects were observed for G577C, T581C, and G609C channels. Significant but smaller effects were also seen for T573C, N578C, Y601C, and I612C; a marginal effect was observed for I598C, which had also shown a marginal conductance effect. The G577C, T581C, and G609C channels were also probed with MTSES and MTS-glucose. Reaction with MTSES increased the selectivity for K^+ over Cl^- , as expected, but the effects were small and in most cases not statistically significant. Reaction with MTS-glucose caused a moderate decrease in K^+ selectivity. Although it is not clear why attaching glucose, via a thioethylurea linker, should have this particular effect on selectivity (or any effect, for that matter), the effect indicates that these residues are part of the selectivity-determining region of the channel, at least after their reaction.

We also examined whether an uncharged MTS reagent placed in this apparently constricted region of the channel could reduce the permeability of a larger cation, TEA^+ . With a salt gradient of 500 mM cis/50 mM trans $TEACl$, pH 7.1, G577C channels gave a V_r of 18 ± 1 mV ($n = 5$), indicating greater permeability for Cl^- than for TEA^+ . Reaction with MMTS or MTS-glucose caused significant conductance decreases, but the effect on V_r was minimal, with at most a 5-mV increase. Similar results were obtained for G609C channels. For comparison, the anion carrier $Hg(C_6F_5)_2$ gave a Cl^- potential of 40 mV. Thus, surprisingly, inserting a bulky group into the channel did not substantially reduce the permeability to TEA^+ .

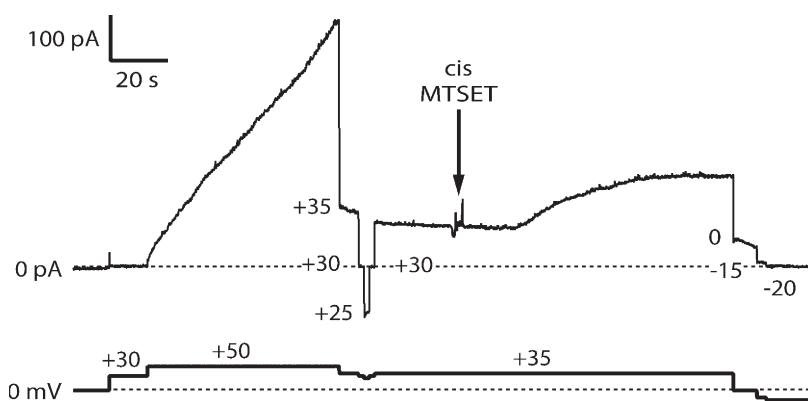


Figure 4. MTSET effect on the ionic selectivity of colicin Ia mutant G577C channels. V_r s were measured with a gradient of 100 mM KCl (cis)/1 M KCl (trans), with the other pH 7.2 buffer ingredients as in Fig. 1. The top and bottom traces, respectively, show the current and voltage versus time. Because of space constraints, some of the numbers giving the voltage (in mV) are placed near the current trace. Approximately 4 min before the start of the record, 135 ng G577C (as well as 0.9 μ g octylglucoside and DTT to 6 μ M) was added to the cis compartment. About 1,500 channels were opened at +50 mV, and the V_r (+30 mV) was determined as the voltage needed to zero the current. This is the normal V_r for colicin Ia channels under these conditions, indicating preferential selectivity for K^+ over Cl^- .

The potential was then held at +35 mV. The addition of 200 μ g MTSET (marked by the arrow) to the cis compartment caused a pronounced increase in the current. This was due not to a conductance increase (the conductance actually decreased), but to a change in the V_r to a negative voltage. By the end of the record, the conductance was too low (because of channel closure at these voltages) to provide a precise value for the V_r , but the measurement of tail currents in subsequent pulses (not depicted) showed it to be $V_r = -18$ mV. Thus, reaction of G577C with MTSET changed the channel selectivity from favoring K^+ permeation to preferring Cl^- .

TABLE II
MTS Effects on the Ionic Selectivity of Colicin Ia Cysteine-mutant Channels

| Mutant | Side of addition | Reagent | V _r (mV) | |
|--------|------------------|-------------|---------------------|----------------|
| | | | Before | After |
| T573C | cis | MTSET | 28 ± 1 (3) | 14 ± 1 (3) |
| G577C | cis | MTSET | 30 ± 0 (2) | -18 ± 2 (2) |
| " | trans | MTSES | 28.5 ± 0.5 (2) | 34 ± 0 (2) |
| " | cis | MTS-glucose | 30 ± 1 (2) | 19 ± 1 (2) |
| N578C | trans/cis | MTSET | 28.8 ± 0.8 (4) | 18.7 ± 0.5 (3) |
| T581C | trans/cis | MTSET | 29.5 ± 0.5 (4) | -24 ± 3 (4) |
| " | trans | MTSES | 29 ± 1 (2) | 30 ± 1 (2) |
| " | trans/cis | MTS-glucose | 28.5 ± 0.5 (2) | 20 ± 0 (2) |
| A582C | trans/cis | MTSET | 28 ± 1 (3) | 25 ± 2 (3) |
| A585C | trans/cis | MTSET | 28 ± 1 (4) | 24 ± 2 (4) |
| F588C | trans | MTSET | 29 ± 1 (3) | 28 ± 1 (3) |
| I598C | trans | MTSET | 27 ± 2 (3) | 22 ± 2 (2) |
| Y601C | trans | MTSET | 29.5 ± 0.5 (2) | 18 ± 2 (2) |
| M605C | trans | MTSET | 31 ± 1 (3) | 27 ± 2 (3) |
| G609C | trans/cis | MTSET | 30 ± 2 (3) | -17 ± 0 (2) |
| " | trans/cis | MTSES | 30 ± 2 (2) | 34 ± 4 (2) |
| " | trans/cis | MTS-glucose | 29 ± 1 (4) | 26 ± 1 (3) |
| I612C | cis | MTSET | 30 ± 1 (3) | 16 ± 2 (3) |
| " | trans/cis | MTSES | 27 ± 1 (3) | 26 ± 3 (3) |

Effects of MTS reagents on the ionic selectivity of colicin Ia cysteine-mutant channels. V_rs were measured before and after MTS addition in the presence of a salt gradient of 100 mM KCl (cis): 1 M KCl (trans), pH 7.2 buffer. The V_r was determined either by zeroing the current (taking into account any non-gating "leak" current) or by finding the voltage at which the decay of the tail currents reversed direction. V_r is given as the mean ± standard deviation, with the number of experiments, n, in parentheses. In many cases (marked "trans/cis"), the MTS reagent was added to both sides of the membrane. For N578C channels after MTSET treatment, the tail currents had two components with different selectivities; the table gives V_r for the faster-closing component, which we estimated to represent about 70% of the conductance at +50 mV. The slower-closing component had V_r = -13.5 ± 0.5 mV (n = 2).

Searching for the End of Helix H9

Because the pattern of reactivity exhibited an α -helical periodicity extending to residue 612 at the end of the hydrophobic segment (see Discussion), we tested the next four residues, representing an additional turn of the putative helix, to see if the helical pattern continued. We found that in 0.1 M KCl, pH 7.2 buffer, cis MTSET had a small effect on D613C and E614C channels; the percentage decreases in macroscopic conductance were 7 ± 1 and 9 ± 4, respectively, with n = 3 experiments for each mutant. S615C and L616C channels were not affected by cis MTSET, and none of the four mutant channels showed any effect of trans MTSET. These results brought the helical pattern to an end (see Discussion).

MTS Effects on Colicin E1

Wild-type colicin E1 has a cysteine at residue 505, in helix H9 of the channel-forming domain (Elkins et al., 1997); using a standard sequence alignment, this is seen to correspond to residue G609 of colicin Ia (Fig. 5). If the colicin Ia and E1 channels have a similar structure, we would expect MTS reagents to have similarly large effects on colicin E1 as on colicin Ia mutant G609C. We found that, indeed, MTSET (unpublished data) and MMTS (Fig. 6 A) caused a large conductance decrease in macroscopic experiments with wild-type colicin E1

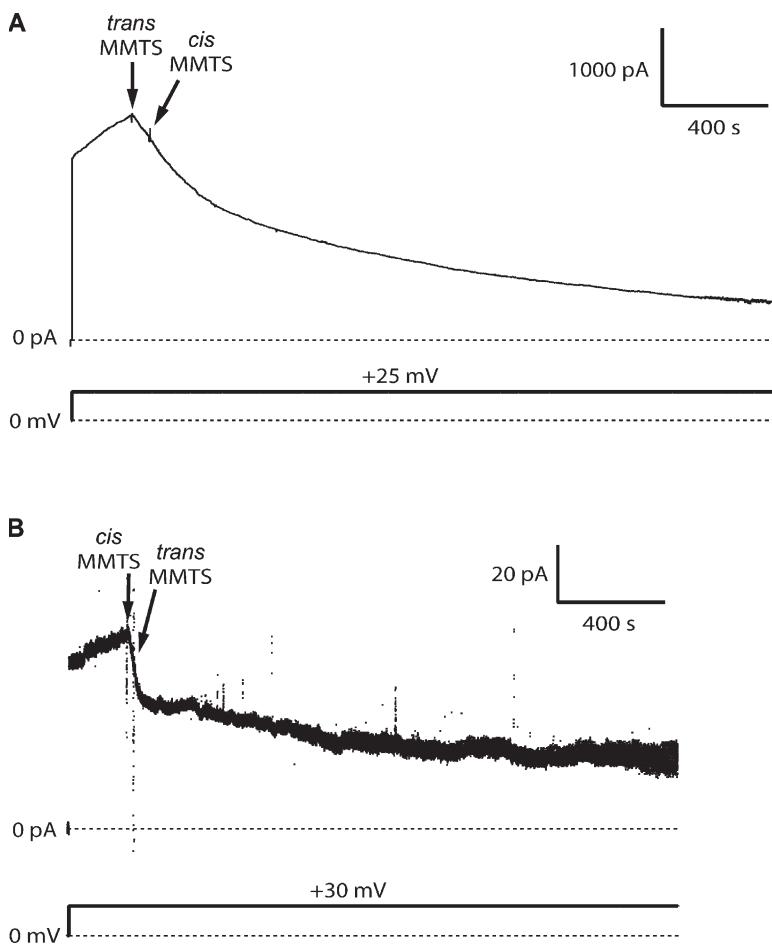
channels. (As a control, we checked that trans and cis MMTS and MTSET had no effect on the channels formed by colicin E1 mutant C505A, which has no cysteine residue.) Fitting the decay as a sum of two exponential components, we obtained percentage decreases in conductance A_f = 28 ± 6 and A_s = 61 ± 9, with respective time constants τ_f = 138 ± 26 s and τ_s = 1,222 ± 164 s (n = 3). (We have defined the parameters so that A_f + A_s equals the total percentage conductance decrease, rather than 100%.) This reaction of MMTS with wild-type colicin E1 was remarkably slow compared with colicin Ia G609C, for which single-exponential fits gave τ = 7 ± 2 s (n = 2). We hypothesized that the slow reaction of MMTS with colicin E1 residue C505 might be due to the proximity of a negatively charged side chain from residue D473. (The corresponding residue in colicin Ia, G577, is not charged; Fig. 5.) That is, the negative charge might shift the pK_a of cysteine 505, so its sulphydryl group would spend less time in the reactive, ionized state. To test this conjecture, we examined the reaction of MMTS with colicin E1 mutant D473N, in which the negatively charged aspartate is replaced by a neutral asparagine. We found that the rate of reaction was much faster (Fig. 6 B). Two-exponential fits gave percentage decreases in conductance A_f = 36 ± 2 and A_s = 29 ± 6, with respective time constants τ_f = 12 ± 2 s and τ_s = 1,231 ± 587 s (n = 3). The main effect of the mutation

| | 570 | 580 | 590 | 600 | 610 | |
|----|---------------------|-------------|-------|-------------|-------------|------------|
| Ia | K TETI IAGNA | ATALV ALVFS | ILTGS | ALGII GYGLL | MAVTG ALIDE | SL |
| Ib | K LESL AAGRA | ASAFT AWAFS | VMLGT | PVGIL GFAII | MAAVS ALVND | K F |
| E1 | T LEKK AADAG | VSYVV ALLFS | LLAGT | TLGFW GIAIV | TGILC SYIDK | NK |
| K | TLEKS AVDVG | VAKIV ALMFS | FIVGV | PLGFW GIAIV | TGIVS SYIGD | D E |
| | ++ | ++ | + | | | ▲ |

the convenience of the reader, these may be converted to colicin E1, K, or Ib numbers by subtracting 104, 78, or 0, respectively.) Plus signs (+) mark the positions in the colicin Ia hydrophobic segment at which one of the other colicins has a charged residue; those residues in colicin Ia are T573, I574, G577, N578, and A582. Position 609, at which colicin E1 has a cysteine residue, is marked by a triangle. The CLUSTAL W algorithm (Thompson et al., 1994) was used to make the alignment; these four colicins are well aligned throughout their C domains, with no gaps required, starting from colicin Ia residue K450. Sequences are from the designated references: E1 (Yamada et al., 1982), Ib (Varley and Boulnois, 1984), Ia (Mankovich et al., 1986), and K (Pils and Braun, 1995).

was to speed up the faster component of the decay by more than a factor of 10. (There was also a significant decrease in the amplitude of the slower component.) It seems likely that the faster component represents the rate of MMTS reaction, which instantly reduces the single-channel conductance, whereas the slower component corresponds to an alteration in gating of the reacted channels. Under this interpretation, MMTS caused quite similar decreases in the open channel con-

ductance of wild-type colicin E1 (28%), D473N (36%), and colicin Ia G609C (35%; see Table I). Furthermore, the time constants of reaction were similar for colicin E1 D473N (12 s) and colicin Ia G609C (7 s), whereas it was slower for wild-type colicin E1 (138 s). These results support our hypotheses that colicins Ia and E1 have similar open channel structures, and that E1 residue D473 is close enough to residue C505 to influence its reactivity.



two exponential components, with amplitudes $A_f = 36\%$ and $A_s = 36\%$, and time constants $\tau_f = 15.5$ s and $\tau_s = 797$ s, respectively. In B, the sampled current points were not connected by lines to make the noise spikes less obtrusive. The solution on both sides of the membrane was as in Fig. 3. The amounts of colicin, octylglucoside, and DTT added to the cis compartment and DTT added to the trans compartment were, respectively, 585 ng, 4.5 μg, 5 μM, and 5 μM in A, and 338 ng, 4.5 μg, 10 μM, and 0 in B.

Figure 5. Sequence alignment of colicin Ia, Ib, E1, and K hydrophobic segments. Charged residues are shown in boldface. The α -helices from the crystal structures of colicins Ia (Wiener et al., 1997) and E1 (Elkins et al., 1997) are underlined. Residue numbers for colicin Ia are shown. (For

DISCUSSION

We have used the substituted cysteine accessibility method (Karlin and Akabas, 1998) to identify channel-lining residues in the colicin Ia hydrophobic segment. A series of mutant colicins was prepared, with each residue (573–612) substituted by a unique cysteine. (Wild-type colicin Ia has no cysteine.) Of these 40 positions, 11 exhibited a step change in single-channel conductance upon reaction with MTSET: T573C, G577C, N578C, T581C, A582C, A585C, F588C, Y601C, M605C, G609C, and I612C (Fig. 3 and Table I). MTSET also caused significant changes in ionic selectivity for seven of these mutant channels (T573C, G577C, N578C, T581C, Y601C, G609C, and I612C; Table II). In particular, G577C, T581C, and G609C channels showed large effects on selectivity. Reaction with MTSES, MMTS, and MTS-glucose also points to these three residues as part of a relatively constricted region of the pore. For most of these mutant channels, the sign of the conductance change caused by MTSET, MTSES, and MMTS was consistent with the attached group having a direct electrostatic or steric effect on the permeant ions. Considering the short Debye length in a 1-M salt solution (3 Å), this indicates that the cysteine residue is located in the channel. The exception was Y601C, for which each of these reagents produced a conductance increase; this presumably reflects an alteration in the channel structure upon reaction. For each reactive mutant, different MTS compounds produced different effects on the channel conductance (Table I). This demonstrates that the MTS compounds acted directly upon the colicin's cysteine residue, as opposed to having some indirect action, such as scavenging the small amount of DTT in

the bath compartment and thereby allowing the cysteine to oxidize.

Secondary Structure of the Colicin Ia Hydrophobic Segment

In the crystal structure of colicin Ia (Wiener et al., 1997), the hydrophobic segment consists largely of an α -helical hairpin formed by helices H8 (580–594) and H9 (597–612) of the C domain. It is natural to ask whether these segments remain helical in the open channel state. In helical-wheel diagrams for H8 and H9, the reactive positions cluster on one face of each helix, suggesting that the answer is yes (Fig. 7). This pattern extends from G577C to F588C in H8 and from Y601C to I612C in H9, in each case a stretch of 12 residues representing three turns of a helix. (We cannot be certain that the cysteine residue in mutant Y601C actually lines the channel, due to the anomalous effects of MTS reaction at this position. However, the kinetics of its response to MTS reagents [unpublished data] were comparable to those of the other mutants listed in Table I, and were much faster than those of its neighbor, G602C, so it seems reasonable to tentatively suppose that Y601 does, in fact, line the channel). This corresponds to a rise of $11 \times 1.5 \text{ \AA} = 16.5 \text{ \AA}$ along the helical axis. We suppose that residues I574 and S589 are outside the limits of H8, in the open channel state, so they do not interrupt the helical face in Fig. 7. Residue V584 lies within the postulated helical region, however, so, in the simplest interpretation, its failure to show a reaction defines one boundary of the water-accessible face of H8. Assuming a simple geometry for the exposure of H8 to the aqueous channel, this implies that residue T573 cannot be part of H8 in the open channel

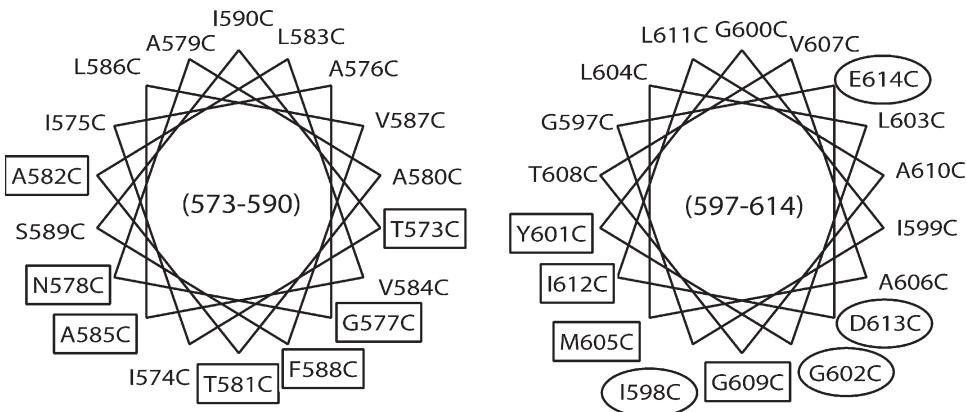


Figure 7. Helical-wheel diagrams showing the pattern of MTS reaction in the colicin Ia hydrophobic segment. The name of each mutant showing an MTS effect on the single-channel conductance is enclosed by a rectangular box. Ovals enclose mutants that showed a less certain effect: a questionable effect on selectivity (I598C), a very slow effect on the macroscopic conductance (G602C), or a small macroscopic conductance effect in 0.1 M KCl buffer (D613C and E614C). The clustering of reactive residues on one face of each helix clearly indicates that these protein segments are α -helical in the open channel state. (This excludes E614C, which presumably lies beyond the end of H9.) The helices are positioned so as to mimic the relative orientation of H8 and H9 in the aqueous crystal structure.

state. (If, alternatively, V584C failed to react due to some local steric factor, such as occlusion by Y601, then T573 might be part of H8, although the lack of an MTS effect on I574C argues against this.)

It is worth mentioning two additional mutants that displayed suggestive, but not conclusive, effects of MTSET. Cis MTSET affected the macroscopic conductance of G602C, even after excess colicin was perfused away, and when a large holding potential was used to discourage channel closing. This could be interpreted as evidence for an effect on the single-channel conductance; however, the extreme slowness of the effect raises suspicion that the reaction might actually have occurred during brief sojourns in the closed state. In selectivity experiments, the reaction of MTSET with I598C appeared to produce a small change in V_r . Although the change was not statistically significant, it is possible that further experiments could show it to be a real effect. In any case, it is not critically important to our channel model whether or not these two mutants had MTS effects; we can say I598 is outside the limits of H9, and G602 is at or near the boundary of the water-exposed face of the helix.

Up to this point, we have not identified the end of helix H9 in the open channel structure. Our macroscopic experiments on mutants D613C through L616C help to provide an answer. Mutant D613C reacts with cis MTSET, but this does not provide much information because residue D613 is located on the boundary of the reactive

face of H9 in Fig. 7; either a reaction or a failure to react could be rationalized in terms of the helical model. If residue E614 were part of H9, it would be located on the opposite face of the helix, relative to the other reactive residues (Fig. 7), so the reaction of E614C with cis MTSET suggests that it is not part of H9. Thus, there is no evidence that H9 extends beyond residue I612 in the open channel state, and there is evidence against its extending as far as residue E614. For both D613C and E614C, of course, single-channel experiments would be required to conclusively show that MTSET reacts in the open channel state; however, because of the small magnitude of the macroscopic effects we did not attempt this. If we consider extending H9 farther, the failure of mutant S615C to show an effect of MTSET is consistent with the helical model. If H9 extended as far as L616, this residue would be located on the reactive face of the helix (at the same position as I598C in Fig. 7); the failure to observe any MTSET effect with L616C suggests that this is not part of the helix.

Orientation of H8 and H9 in the Membrane

Since the first crystal structure of a colicin C domain was determined (Parker et al., 1989), most researchers have accepted that the H8-H9 α -helical hairpin inserts in a transmembrane orientation, with the H8-H9 loop moving to the trans side of the membrane. A mutational study of colicin E1 provided convincing evidence that the hydrophobic segment maintains a hairpin structure

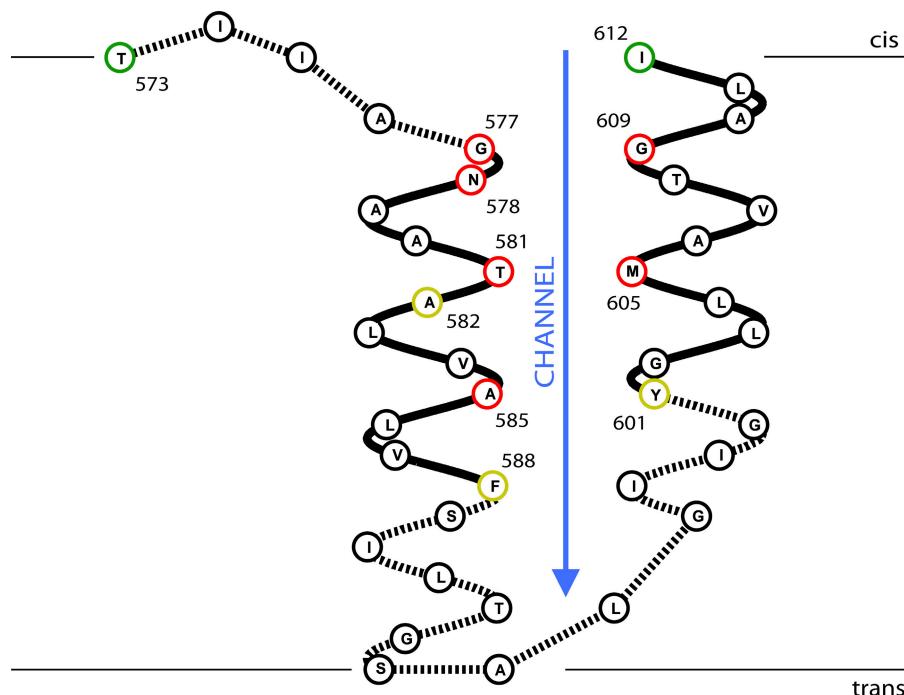


Figure 8. Schematic model of the colicin Ia hydrophobic segment in the open channel state. The effect of MTS reagents on the single-channel conductance of each cysteine mutant is coded as follows: green, effect of cis MTS only; red, both cis and trans MTS effects; yellow, trans MTS effect only; black, no effect. Residues in the inferred α -helical segments are connected by thick solid lines. Residues in other regions are connected by dotted lines to indicate our ignorance of the secondary structure there. Residues A580-S594 and G597-I612 are arranged in an α -helical pattern for comparison with their conformation as helices H8 and H9 of the aqueous crystal structure (Wiener et al., 1997); residues G577-A579 have been added to H8 on the basis of our results; and residue A576 was also included in H8 for good measure. The distance between the thin horizontal lines indicates the 30-Å thickness of the hydrocarbon region of a typical bilayer membrane. For illustrative purposes, the reactive faces of H8 and H9 are shown facing each other, but really we just mean to imply that they face the aqueous channel.

during channel formation *in vivo* (Song et al., 1991). Our model for the colicin Ia hydrophobic segment, based on the accessibility of an attached biotin to streptavidin, placed residue 594 on the trans side and residue 577 on or near the cis side of the membrane in the open channel state, as well as in at least one closed state (Kienker et al., 1997; Jakes et al., 1999). In the present work, the pattern of reaction with cis and trans MTSET supports and refines this model. Mutants T573C and I612C react only with cis MTSET; G577C, N578C, T581C, A585C, M605C, and G609C react from either side; and A582C, F588C, and Y601C react only with trans MTSET.

Fig. 8 shows our model for the secondary structure and orientation of the hydrophobic segment in the open channel state. The residues that (when mutated to cysteine) showed an effect of MTS reagents on the single-channel conduction properties are colored according to their accessibility to cis or trans reagent. For the most part, the arrangement of reactive residues makes sense, with those near the cis interface reacting only with cis MTS, those in the middle reacting with either cis or trans MTS, and those near the trans interface reacting only with trans MTS. The main anomaly is that A582C reacts only with trans MTS, whereas A585C, which should be nearer to the trans side, reacts from either side. Perhaps this could be explained if A582C is actually not exposed to the pore, but instead is accessible from the trans side via an aqueous crevice that does not extend to the cis side.

We cannot specify the secondary structure of the segment between H8 and H9, that is, residues S589 through G597, because MTSET reaction at these positions does not appear to affect the single-channel conductance or selectivity. At several of these positions, however (S589C, G593C, S594C, and G597C in particular), reaction with trans MTSET appeared to increase the number of open channels, most likely by trapping the H8-H9 hairpin in a transmembrane orientation, thereby increasing the opening rate (compare with Kienker et al., 1997). This implies that these residues either line the channel or are otherwise exposed to the trans solution.

Our model for helices H8 and H9 in the open channel state has some similarity to their arrangement in the aqueous crystal structure (Wiener et al., 1997). The reactive positions fall not only on one face of each helix, but essentially on one side of the helical hairpin from the crystal structure (Fig. 9). This suggests that the two helices may remain packed together when the channel forms. Whether they retain their relative position in the axial direction is not so clear. Our results with MMTS suggest that residues 577 and 609 are both located in the narrowest part of the pore. If these two residues are in register, H8 and H9 would have to shift by two helical turns, relative to the crystal structure, which has residue 609 in register with 585.

Shape of the Pore

As the site of reaction is moved from the cis side toward the trans side, there appears to be a smooth trend in the magnitude of MTS-induced changes in the single-channel conductance: first, for a short stretch, there is an increase in magnitude (from I612C to G609C in helix 9) and then a decrease in magnitude over a longer stretch of protein (from G577C to T581C to A585C to F588C in helix 8, or from G609C to M605C in helix 9). (We are only counting the residue that gives the largest MTS effect in each helical turn, so 578C and 582C are neglected here. 601C is also omitted because its reaction appears to alter the channel structure.) This suggests that the pore may have an hourglass shape, with the narrowest part near residues G577 and G609. Our model places this constricted region near the cis end of the pore (Fig. 8). We note that this is the opposite of a previously proposed model for the colicin Ia channel, based on the effects of nonelectrolytes on the single-channel

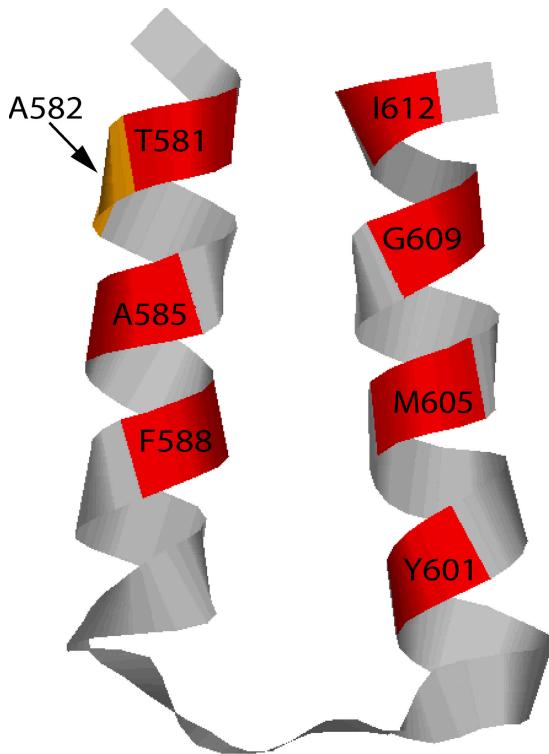


Figure 9. MTS-reactive positions are located on one face of the H8-H9 hairpin in the aqueous crystal structure. The figure shows the hairpin consisting of residues 580–612, plus the flanking residues 579 and 613 (in a truncated rendering). The residues colored red or orange are those that, when mutated to cysteine, evoke a single-channel conductance change upon reaction with MTS derivatives. (Residue A582 is colored orange to distinguish it from residue T581.) Three additional reactive positions, T573, G577, and N578, are not shown because they are not part of the hairpin in the crystal structure. Residues 579–613 were excerpted from the crystal structure of whole colicin Ia (Wiener et al., 1997), available from the Protein Data Bank (Berman et al., 2000) as file 1CII, and displayed as a ribbon model using RasMol (v2.7.1).

conductance (Krasilnikov et al., 1998), which places the constricted region nearer the trans end of the channel (75% of the distance from cis to trans). Conceivably, the two models could be reconciled if we found some additional material to form a wide cis-side entrance, such as the other transmembrane protein segments (H1 and H6–H7) or membrane lipids.

As mentioned above, the reactive stretch in each of the putative helices, residues 577–588 and 601–612, corresponds to a length of 16.5 Å along the helical axis. If we include residue 598, that would extend one helix to 21.0 Å. Either way, this is much too short to span the typical 30-Å thickness of the bilayer hydrocarbon region. One possible explanation is that helices H8 and H9 extend farther into the loop region (residues 589–597), as drawn in Fig. 8, but we do not detect MTS reactions here because the pore is too wide.

It appears that the cysteine residues of G577C, T581C, and G609C occupy a relatively narrow region of the pore, given the substantial conductance decreases attributable to the steric effects of reaction with MMTS, MTS-glucose, and MTSES. Hence, one might reasonably expect that such a reaction would completely block the permeation of a larger ion. Contrary to this expectation, we found that reaction of G577C and G609C with MMTS or MTS-glucose had little effect on the permeability of TEA^+ , relative to that of Cl^- . A possible explanation is that, as suggested previously (Slatin et al., 2008), the channel has both small- and large-diameter open states. The predominant small-diameter state would mostly determine the single-channel conductance, whereas the rarer large-diameter state would account for the low permeability to large ions. (In single-channel experiments, however, we have not been able to identify such states directly, suggesting that the rate of transition between the states would have to be too fast for our recording system to resolve.)

In this context, we note some intriguing parallels between colicin and P2X receptor channels. The selectivity of P2X receptor channels appears to change with prolonged activation by ATP: only small ions permeate initially, but later larger ions such as *N*-methyl-*D*-glucamine can pass (Egan et al., 2006). Whether this phenomenon is due to the dilation of a single pore or the gating of separate conductive pathways has not been settled. There is also evidence that the trimeric P2X receptor channel is lined only by the second transmembrane segment, TM2, for a total of three transmembrane segments per channel (Li et al., 2008), similar to the low-conductance state of the colicin Ia C domain channel (Kienker et al., 2000).

Comparison with Other Colicins

Fig. 5 shows an amino acid sequence alignment of the colicin Ia hydrophobic segment with those of colicins Ib, E1, and K. These colicins are well aligned throughout their entire C domains, so we can reasonably imagine

that their channel structures may be essentially identical. A preliminary way to test this hypothesis is to examine which residues in the colicin Ia–uncharged segment correspond to charged residues in other colicins. We would expect the charged side chains to reside in an aqueous environment, and hence the corresponding positions in colicin Ia should be accessible to MTS reagents. The residues in colicin Ia are T573, I574, G577, N578, and A582. Four of the five are positions at which the single-channel conductance is sensitive to MTS reaction. Our model places the exception, I574, in a loop between H8 and the upstream segment, where it would be exposed to the cis solution. Thus, we conclude that all of these positions are exposed to an aqueous milieu in the colicin Ia channel, in support of the hypothesis. A more direct approach is to test positions in the other colicins for MTS reaction. The conspicuous starting point is C505 of wild-type colicin E1, which corresponds to G609C in colicin Ia. We found that MMTS caused a large conductance decrease in macroscopic experiments with wild-type colicin E1 (Fig. 6 A). In contrast to colicin Ia mutant G609C, however, the reaction rate was very slow. With colicin E1 mutant D473N channels, on the other hand, MMTS caused a fast conductance decrease, comparable in rate and magnitude to its effect on colicin Ia G609C (Fig. 6 B). Thus, the slow reaction of wild-type E1 was due to the influence of D473. Most likely, it raises the pK_a of C505 by an electrostatic mechanism, so the cysteine sulfhydryl group spends less time in the ionized state with which MTS compounds prefer to react (Roberts et al., 1986). This indicates that colicin E1 residues D473 and C505 are close together in the open state of the channel, even though they are 24 Å apart in the crystal structure (Elkins et al., 1997). By an independent argument, involving the large steric effects of MTSES and MMTS on channel conductance, we arrived at a model for the colicin Ia channel that has the corresponding residues G577 and G609 located close together; this lends support to the idea of a precise structural homology between colicin Ia and E1 channels.

It is a curious result that reaction of colicin Ia G577C and G609C channels with MMTS causes a substantial decrease in single-channel conductance (>30%), but the mutation itself, which replaces glycine with cysteine, does not have a measurable effect on the conductance, relative to that of wild-type channels. It is as if the pore diameter is fluid while the channel is forming, with a preferred range of sizes, but the diameter becomes fixed once the channel has formed. This picture is consistent with the fact that the single-channel conductance varies considerably from channel to channel, but the conductance for an individual channel is stable, aside from temporary flickers to other conductance states. This may be related to the observation that colicin E1, labeled (in 6 M urea) at residue C505 with 5, 5'-dithiobis (2-nitrobenzoic acid), can subsequently induce Cl^- efflux with

the same kinetics as unmodified channels (Bishop et al., 1986). Because we have shown that C505 is part of the channel lining, in an apparently constricted region of the pore, it is surprising that attaching such a bulky group did not have a measurable effect.

Comparison with Diphtheria Toxin (DT) T Domain

It has been previously noted that the channel-forming colicin C domain and the DT T domain show similarities in both aqueous crystal structure (Choe et al., 1992) and membrane channel topology (Kienker et al., 2000; Slatin and Kienker, 2003); it is even possible to form functional channels from chimeric proteins with the hydrophobic segments swapped (unpublished data). Because of this, we are obliged to compare our present results on colicin Ia with an earlier, single-channel study of MTS effects on DT T domain cysteine mutants (Huynh et al., 1997). Although it still burns fiercely in our memories, we review, for the reader's sake, the unusual pattern of reactive residues in the DT T domain hydrophobic segment: a stretch of 13 residues with 11 reacting, five non-reacting residues, a stretch of 13 residues with 12 reacting, and 17 nonreacting residues. This pattern does not suggest either an α -helical or a β -sheet secondary structure.

Although the pattern we have seen in the colicin Ia hydrophobic segment is quite different, the lengths of the reactive stretches are actually rather similar: a stretch of 12 residues, 577–588, with some reacting, a loop of 12 nonreacting residues, 589–600, and a stretch of 12 residues, 601–612, with some reacting. It is tempting to conjecture that the reactive stretches in DT T domain have an α -helical structure similar to that in colicin Ia channels. The unusual pattern of reaction might be explained if two channel states of DT (for instance, the open state and the brief flicker-closed state) were imperfectly resolved, and the transition between these states involved a rotation of the helices about their axes. Thus, one face of each helix might be exposed to the aqueous channel, and hence react with MTS reagents, in the open channel state, whereas the opposite face might be exposed in the flicker-closed state.

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