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A Carboxy-Terminal Fragment of Colicin Ia Forms Ion Channels

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Abstract. A carboxy-terminal, 18 kD fragment of colicin Ia, a bacterial toxin, forms ion channels in artificial phospholipid bilayers. This fragment, which comprises a quarter of the intact 70 kD molecule, is resistant to extensive protease digestion and probably constitutes a structural domain of the protein. The ion channels formed by the 18 kD fragment are functionally heterogeneous, having conductances that range from 15 to 30 pS at positive voltages and from 70 to 250 pS at negative voltages, and open lifetimes that range from at least 25 msec to 5 sec. In contrast, ion channels formed by whole colicin Ia open only at negative voltages, at which their conductances range from 6 to 30 pS, and their open lifetimes range from 1 sec to 3 min. Additionally, the open state of the 18 kD fragment channel is characterized by noisy fluctuations in current, while the open state of the whole molecule ion channel is often marked by numerous, stable subconductance states. Since the properties of the fragment channel differ substantially from those of the whole molecule channel, we suggest that portions of the molecule outside of the 18 kD fragment are involved in forming the whole molecule ion channel.

Key words: Colicin Ia fragment — Artificial lipid bilayers — Ion channels — Proteolysis

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Introduction

Ion channel proteins are significant transducers of signals across membranes. To understand the structural basis for ion channel formation and function, we have studied the bacterial toxin colicin Ia, a member of the ion channel-forming family of colicins. This family includes colicins A, B, E1, Ib, K and N as well (*for review see* Cramer et al., 1990; Pattus et al., 1990). The colicins are plasmid-encoded, soluble proteins of 42 to 70 kD which are synthesized upon stress during the SOS response (Morlon et al., 1983; Waleh & Johnson, 1985; Mankovich, Hsu & Konisky, 1986; Salles, Weisemann & Weinstock, 1987). These proteins are released into the media, bind to an outer membrane receptor of target bacteria, and translocate across the periplasmic space to the inner plasma membrane, where they form lethal, transmembrane ion channels. Colicin ion channels are voltage-gated and relatively nonselective, as determined by recordings from artificial membranes (Schein, Kagan & Finkelstein, 1978; Weaver et al., 1981; Seta et al., 1983; Raymond, Slatin & Finkelstein, 1985).

Proteolytic digestion (Carmen Martinez, Lazdunski & Pattus, 1983) and genetic truncation (Baty et al., 1988; Baty et al., 1990) of colicin A have identified the carboxy-terminal third of the protein as the ion channel-forming region. Similarly, proteolytic cleavage of colicin E1 has shown that the carboxy-terminal third of colicin E1 is responsible for forming ion channels (Dankert et al., 1982; Ohno-Iwashita & Imahori, 1982). The functional properties of the ion channel-forming domains of colicins A and E1 are generally similar to those of each respective whole molecule, although some differences do exist in voltage dependence, gating kinetics, and ion selectivities (Carmen Martinez et al., 1983; Cleveland

et al., 1983). These functional ion channel-forming regions appear to be equivalent to structural domains. The x-ray crystal structure of the ion channel-forming portion of colicin A reveals that it is a compact bundle of 10 α-helices (Parker et al., 1989; Parker et al., 1990), and nuclear magnetic resonance (NMR) shows that the ion channel-forming portion of colicin E1 is structurally similar to the colicin A fragment (Wormald et al., 1990).

We asked whether a carboxy-terminal portion of the 70 kD protein colicin Ia is also capable of forming ion channels. We report that a tryptic, carboxy-terminal fragment of colicin Ia does form ion channels, but that these channels differ substantially from those formed by intact colicin Ia. These results are discussed in the context of other channel-forming colicins and their channel-forming fragments.

Materials and Methods

Probing the Structure of Colicin Ia with Trypsin and V8

Colicin Ia was expressed and purified (Mel & Stroud, 1993) from plasmid pJK5 in *Escherichia coli* strain 294 (Weaver, Redborg & Konisky, 1981). The purified molecule was digested at 37°C with bovine trypsin (Worthington) at a 1:3 trypsin:colicin Ia molar ratio, with colicin Ia at a concentration of 270 μM in 50 mM NaCl, 20 mM citrate buffer, pH 5.2. The reaction was stopped by the addition of leupeptin to a final concentration of 90 μM.

For digestion with *Staphylococcus aureus* V8 protease (Endoproteinase Glu-C, Boehringer Mannheim), the reaction was carried out at 37°C at a V8:colicin Ia molar ratio of 1:3, with colicin Ia at a concentration of 44 μM in 50 mM phosphate buffer, pH 7.8, and 2.4 M urea. The products of both reactions were examined by SDS-PAGE (Laemmli, 1970).

Purification of Fragments

Purified colicin Ia was digested with trypsin as above, except at a lower trypsin:colicin Ia molar ratio of 1:30. The products of the digest were purified by size-exclusion HPLC on two, tandem Biosil TSK 250 columns (each 21.5 mm × 60 cm), which had been equilibrated with 50 mM NaCl, 20 mM citrate buffer, pH 5.2. Three resolved fractions, which eluted at 117, 141, and 160 min at a column flow rate of 2 ml/min, were collected and subjected to amino-terminal sequencing and relative molecular weight determination by SDS-PAGE. Only the leading edge of the peak eluting at 160 min was collected, since the trailing edge of this peak overlaps with another peak.

Formation of Artificial Phospholipid Bilayers

Artificial phospholipid bilayers were formed on the tip of tight-seal pipettes (Coronado & Latorre, 1983; Suarez-Isla et al., 1983) as described in Ghosh and Stroud (1991) using soybean asolectin

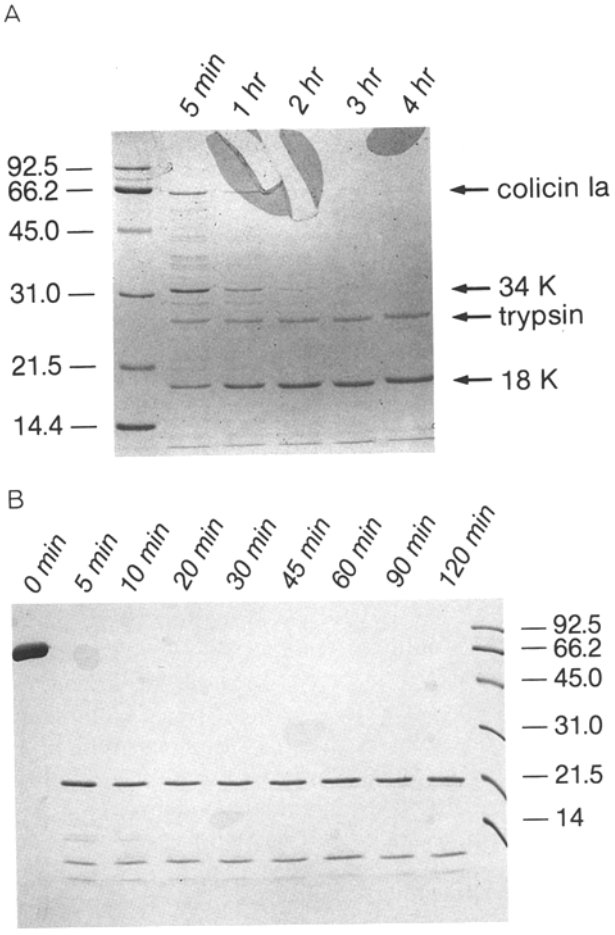

Fig. 1. Proteolytic digestion of colicin Ia. (*A*) 14% SDS-PAGE of tryptic digest of colicin Ia. Colicin Ia was digested with trypsin at a trypsin:colicin Ia molar ratio of 1:3 with colicin at 270 μM at 37°C, and time points were collected as indicated on the figure. The 18 kD colicin Ia fragment, whose amino-terminus is residue 451, persists for 4 hr, while the 34 kD fragment, whose amino-terminus is residue 7, persists for 1 hr. The band at 28 kD corresponds to trypsin. (*B*) 16% SDS-PAGE of V8 digest of colicin Ia in 2.4 M urea. Colicin Ia was digested with V8 at a V8:colicin Ia molar ratio of 1:3 with colicin Ia at 44 μM at 37°C, and time points were collected as indicated on the figure. A 20 kD fragment, whose amino-terminus is residue 446, and several lower molecular weight fragments persist through 2 hr of digestion.

(Associated Concentrates) which had been purified (Kagawa & Racker, 1971). This procedure yielded membrane-sealed tips having a resistance of 5–100 GΩ with a success rate of 80–90%. Colicin Ia and the tryptic fragments were added to the unstirred, 2 ml bath solution containing the membrane-sealed tip to a final concentration ranging from 0.03–145 μg/ml. Ion channels typically appeared 10 to 20 min after the introduction of the protein into the bath solution.

Channel Recordings

A voltage-clamp amplifier (EPC-5, List Electronics) was used to set the transmembrane potential and to measure ionic currents. Ground potential was defined as that of the bath, so the reported

potentials represent those of the *trans* compartment; the *cis* compartment is defined as the compartment to which protein was added. The sign convention is such that current into the pipette (from *cis* to *trans*) is defined as positive and shown as an upward deflection. Ion channels were recorded with *cis* and *trans* compartments containing symmetrical solutions of either 500 mM or 1,000 mM NaCl and 1 mM $CaCl_2$, 10 mM dimethylglutarate, pH 4.0. Current and voltage records were recorded on chart paper with a Gould 220 brush recorder resulting in 40 Hz filtering of data.

Results

The Carboxy-Terminal Quarter of Colicin Ia Is Resistant to Proteolysis

Tryptic digestion of colicin Ia yields two major products, a protease-resistant fragment of M_r 18.0 ± 0.4 kD (n = 7) and a less resistant fragment of M_r 34.1 ± 1.0 kD (n = 6) (Fig. 1*A*). The 34 kD fragment disappears after 2 hr of digestion at 37°C at a trypsin : colicin Ia molar ratio of 1 : 3, while the 18 kD fragment persists for at least 4 hr. The amino-terminus of the 34 kD fragment is residue 7, having been cleaved at arginine 6, as determined by amino-terminal sequencing. Its carboxy-terminus probably extends to arginine 313, as determined from its relative molecular weight and the specificity of trypsin for lysines and arginines. However, given the errors in calculating the relative molecular weight, it is possible that either arginine 299 or arginine 313 could be the carboxy-terminus of the 34 kD fragment. The 18 kD fragment begins at aspartate 451 and likely extends to the carboxy terminus of the intact molecule, isoleucine 626. However, the fragment may be shorter, having been cleaved at either arginine 619 or lysine 622. Even with the uncertainty in the carboxy-termini of these fragments, it is clear that the 34 kD fragment constitutes the amino-terminal half of colicin Ia while the 18 kD fragment constitutes the carboxy-terminal quarter.

The structural stability of the carboxy-terminal quarter is confirmed by a more rigorous digest in the presence of 2.4 M urea using V8 (Fig. 1*B*). After 5 min of digestion at 37°C at a V8 : colicin Ia molar ratio of 1 : 3, only a 20.4 ± 0.2 kD M_r (n = 4) fragment as well as several lower molecular weight fragments remain. The 20 kD fragment, whose amino-terminus is residue 446 and whose carboxy-terminus probably extends to the carboxy-terminus of intact colicin Ia, remains uncleaved for at least 2 hr. The 20 kD V8 fragment is longer by six residues at its amino-terminal end and probably by seven residues at its carboxy-terminal end than the 18 kD tryptic fragment. Therefore, as determined from tryptic and V8 proteolysis, the carboxy-terminal quarter of colicin Ia appears to constitute a stable, structural domain.

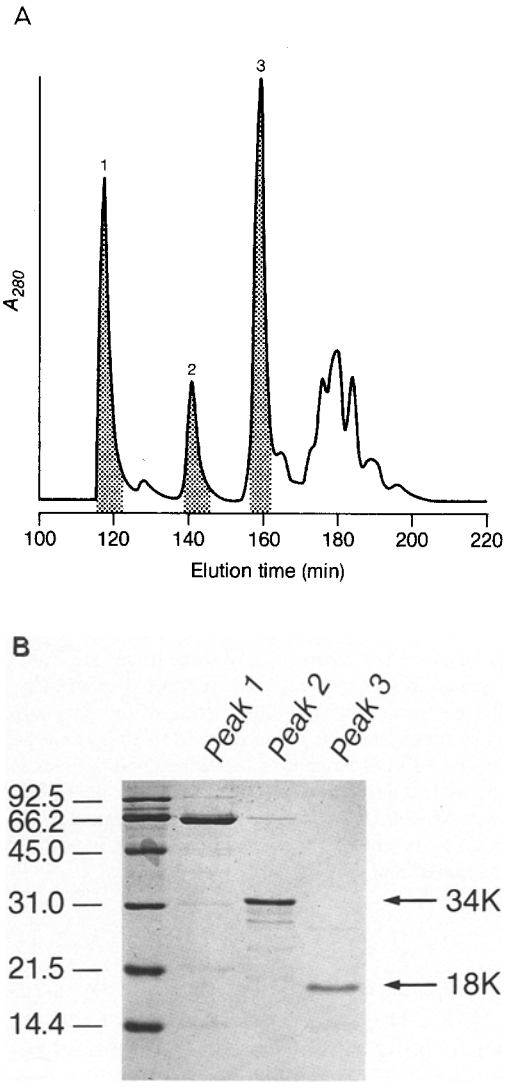

Fig. 2. Tryptic digestion and purification of 34 and 18 kD fragments. (*A*) Size exclusion HPLC of tryptic digest of colicin Ia. The extent of the fractions collected are indicated by shading. Digestion conditions were as in the legend to Fig. 1*A* except that the trypsin : colicin Ia molar ratio was 1 : 30. The digest was stopped at 4 hr, and the products were applied to two, tandem Biosil TSK 250 columns at a flow rate of 2 ml/min. The peaks eluting are: Peak *1*, colicin Ia at 117 min; Peak *2*, 34 kD fragment at 141 min; and Peak *3*, 18 kD fragment at 160 min. (*B*) 16% SDS-PAGE of peak fractions from size exclusion HPLC. Lane 1 contains 1.1 μg of *Peak 1*, 117 min; lane 2 contains 0.6 μg of *Peak 2*, 141 min; and lane 3 contains 0.7 μg of *Peak 3*, 160 min.

The 18 kD Colicin Ia Fragment Forms Ion Channels

To determine whether the carboxy-terminal quarter of colicin Ia possesses ion channel-forming activity,

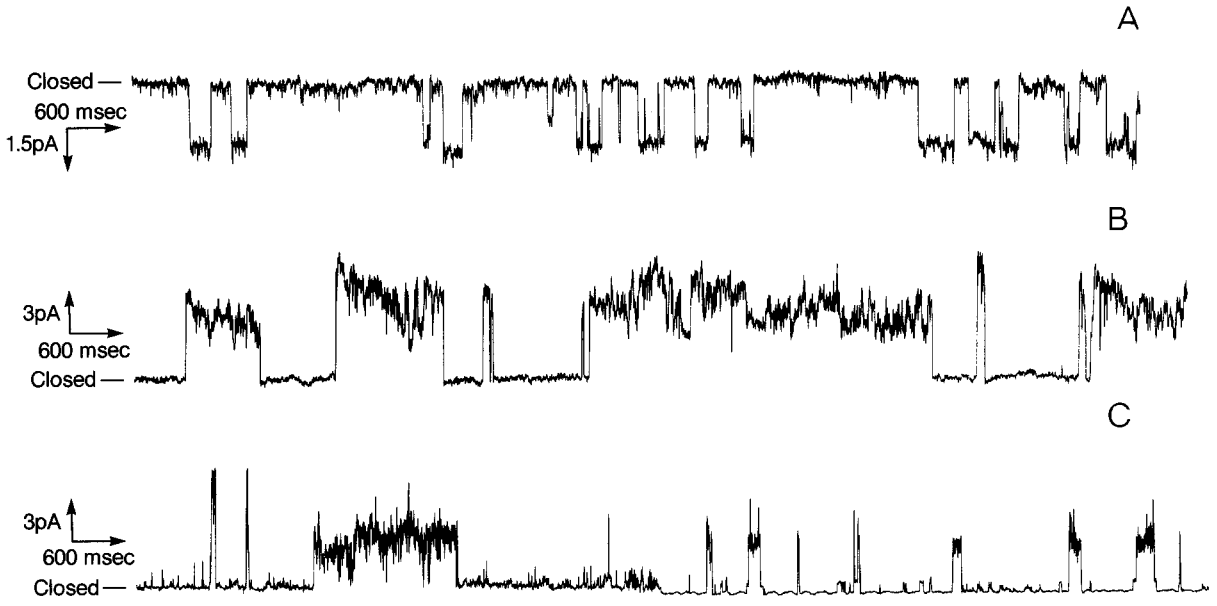

Fig. 3. 18 kD fragment ion channels. The three traces are from three different membranes. (*A*) Single channel currents resulting from the 18 kD fragment in symmetrical 500 mM NaCl, 1 mM $CaCl_2$, 10 mM dimethylglutarate, pH 4.0, at a holding potential of +78 mV. The horizontal bar represents the closed state of the channel, and the downward deflections indicate opening of the channel. The conductances of these channels range from 15 to 30 pS and their open times range from 25 to 400 msec. (*B*) Single channel currents resulting from the 18 kD fragment in the same conditions as in 3*A*, except that the holding potential is −55 mV. The conductance of these channels is approximately 100 pS and their open times range from approximately 200 msec to 4 sec. Large fluctuations in current flowing through the open channel are evident. (*C*) Single channel currents resulting from the 18 kD fragment in the same conditions as in *A*, except that the holding potential is −45 mV. The channels displayed range between approximately 60 and 180 pS, although smaller channels of 10 pS are evident.

the 18 kD tryptic fragment was purified by size-exclusion HPLC (Figs. 2*A* and *B*) and assayed for ion channel-forming activity by single channel recording techniques. The addition of the 18 kD, carboxy-terminal fragment to artificial phospholipid bilayers results in the appearance of ion channels which are characterized by noisy open states (Figs. 3*A–C*). The current flowing through these channels is not stable, but instead fluctuates at a rate greater than the 40 Hz resolution limit of our recording system. These fluctuations can be as great as the open channel current. Furthermore, ion channels formed by the 18 kD fragment are functionally heterogeneous such that a range of conductance rather than a single conductance value is observed. The range of conductance is dependent on the polarity of the voltage. At positive voltages, the conductance ranges from 15 to 30 pS in 500 mM NaCl, pH 4.0, as determined from 17 different membranes and 153 channels. However, at negative voltages, the conductance is higher, ranging from 70 to 250 pS, as determined from 21 different membranes and 204 channels. Typical single channel currents arising from channels formed by the 18 kD fragment at positive voltages are shown in Fig. 3*A* and from negative voltages in Fig. 3*B* and *C*. Infrequently, channels of much lower conductance, approximately 10 pS, are observed at negative voltages (Fig. 3*C*). At both positive and negative voltages, channels that are unresolved by the 40 Hz limit of our recording system are observed, indicating that open lifetimes can be as short as at least 25 msec. The open lifetimes of these fragment channels are observed to range to approximately 700 msec at positive voltages and to approximately 5 sec at negative voltages.

To determine whether these ion channels result uniquely from the action of the 18 kD fragment and whether other portions of the molecule are capable of forming ion channels, the amino-terminal 34 kD fragment was also assayed for ion channel-forming activity. This fragment was generated by the same tryptic digest yielding the 18 kD fragment and purified identically to the 18 kD fragment (Fig. 2*A* and *B*). In contrast to the 18 kD fragment, the 34 kD fragment does not form detectable ion channels (>1 pS) within the 6 hr duration of an experiment (*data not shown*). Therefore, it appears that only the carboxy-terminal quarter of colicin Ia is required to form ion channels.

Fig. 4. Whole colicin Ia channels. The two traces are from two different membranes. (*A*) Single channel currents resulting from whole colicin Ia in symmetrical 500 mM NaCl, 1 mM $CaCl_2$, 10 mM dimethylglutarate, pH 4.0, at a holding potential of −93 mV. The current was filtered at 10 Hz with an 8-pole Butterworth filter resulting in the low current noise level in this trace. The horizontal bar represents the closed state of the channel, and upward deflections represent channel openings. At the beginning of the record, the closure of a previously open channel is followed by the opening of another channel or the same channel to a subconductance state. Various conductance states are evident with the lowest conductance state being 11 pS. (*B*) Single channel currents resulting from whole colicin Ia in the same conditions as the legend to Fig. 4*A*, except that the holding potential is −80 mV. A previously open channel transits through various subconductance states and finally closes from the second largest subconductance state, which is 25 pS.

Channels Formed by the 18 kD Fragment Are Dissimilar to Channels Formed by Whole Colicin Ia

Ion channels formed by the 18 kD fragment, however, differ considerably from those formed by whole colicin Ia. As determined from 10 different membranes and 28 different channels, the conductance of whole colicin Ia at negative voltages in 500 mM NaCl, pH 4.0 ranges from 6 to 30 pS, approximately 10-fold less than that for the 18 kD fragment at negative voltages. Typical single channel currents arising from channels formed by whole colicin Ia are shown in Fig. 4*A* and *B*. A single conductance value is difficult to assign to these channels, since a variety of conductance states are found (Fig. 4*A* and *B*). Some of these are subconductance states of the same channel rather than separate conductance states of a number of channels. This was determined from the observation that closure to the zero current level often occurs from a state other than the lowest conductance state. One channel was observed to vary among several states and then to close from the second largest subconductance state (Fig. 4*B*); the lifetime of each of these subconductance states is on the order of seconds. The open lifetime of the whole colicin Ia channel ranges from 1 sec to 3 min, contrasting with the much shorter lifetimes of the 18 kD fragment.

In further contrast, the opening of the colicin Ia channel but not of the 18 kD fragment is dependent on voltage. Membranes in which several whole colicin Ia channels are active show that the channel conducts current only at negative voltages (Fig. 5*A* and *B*). Whole colicin Ia channels close at positive voltages but reversibly reopen at negative voltages. In contrast, the 18 kD fragment conducts ions at either positive or negative voltages (Fig. 3*A–C*), although its conductance is diminished at positive voltages.

Discussion

We have found from proteolytic digestion and single channel recordings that the carboxy-terminus of colicin Ia is capable of forming ion channels, as are the carboxy-terminal portions of colicins A and E1. However, in contrast to colicins A and E1, the ion channel properties of this colicin Ia carboxy-terminal fragment differ greatly from those of the whole molecule.

The ion channel properties found in this and other studies (Bullock & Cohen, 1986; Nogueira & Varanda, 1988) for whole colicin Ia are generally similar to those found for whole A and E1. In comparison to the range of 6 to 30 pS found for colicin Ia in 500 mM NaCl, pH 4.0, colicin A has a conductance of 13 pS in 1 M KCl, pH 6.2 (Carmen Martinez et al., 1983), and colicin E1 has a conductance of 24 pS in 1 M KCl, pH 4 (Raymond, Slatin & Finkelstein, 1985). The recordings in this study were carried out at pH 4 since the membrane-association and channel-forming activities of colicins are greatest at pH values ≤ 4 (Bullock et al., 1983; Pattus et al., 1983; Davidson et al., 1984; Davidson et al., 1985; Mel & Stroud, 1993). The finding that the whole colicin Ia channel can adopt a number of subconductance states with lifetimes in the range of seconds has also been noted for colicin E1 (Raymond et al., 1986). In general, channels formed by whole colicins A, E1, and Ia have similar conductances, a dependence

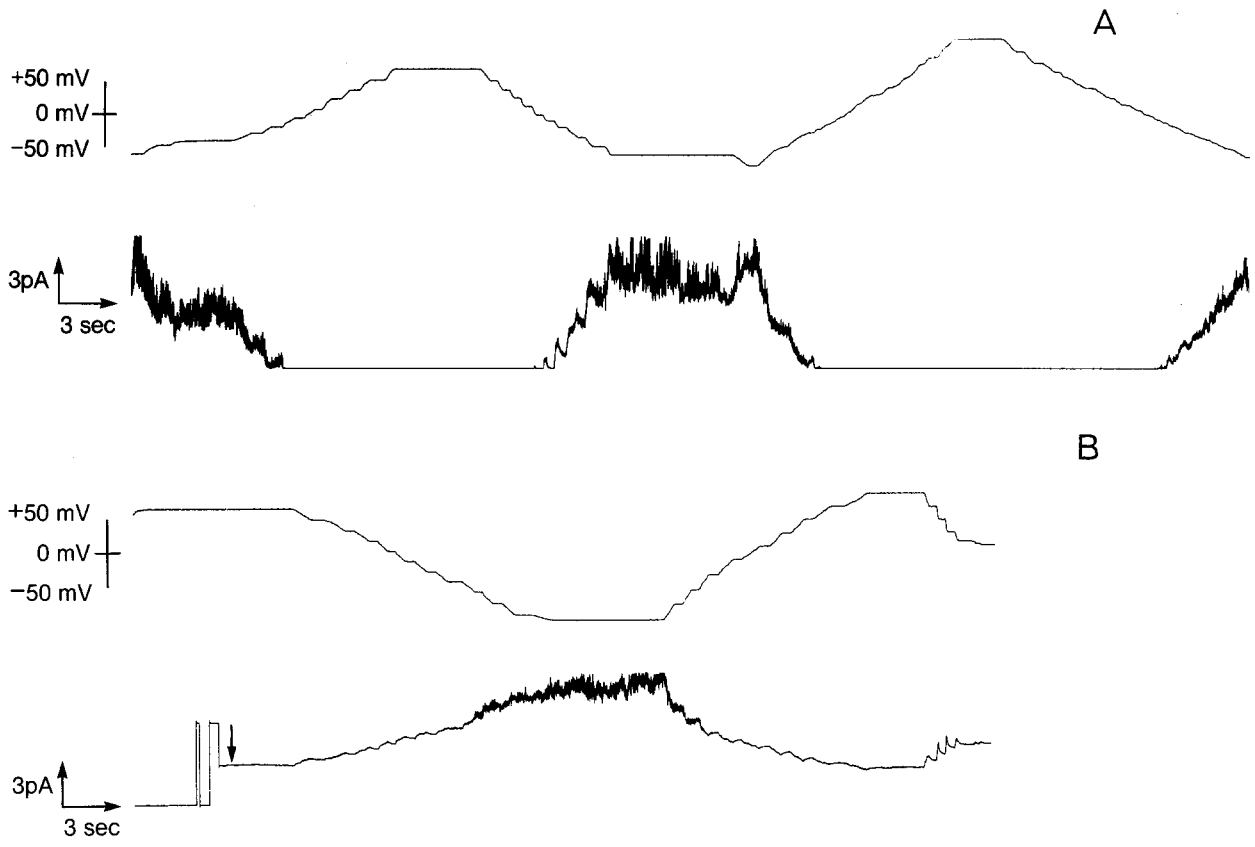

Fig. 5. Whole colicin Ia opens only at negative voltages. The two traces are from the same membrane. (*A*) Single channel currents resulting from whole colicin Ia in symmetrical 1 M NaCl, 1 mM $CaCl_2$, 10 mM dimethylglutarate, pH 4.0. The voltage was ramped between +75 and −80 mV. About 5 to 20 channels are open, resulting in a peak total conductance of 140 pS at −80 mV. Upward deflections at negative voltages, which represent channel openings, are evident. The current at positive voltages is saturated, and therefore an offset to the amplifier was applied in (*B*), shown at the arrow. Here, current at positive voltages is no longer saturated, although current at negative voltages is partially saturated. This record shows that no channel activity is found at positive voltages while upward deflections, indicative of channel openings, are still found at negative voltages.

on negative voltage for opening, and open lifetimes in the range of seconds to minutes.

However, unlike colicins A and E1, colicin Ia's channel-forming fragment has ion channel properties which differ markedly from those of the whole molecule. The single channel conductance of the 18 kD fragment is 10-fold greater at negative voltages than that of the whole molecule, and the open lifetimes are much shorter. Therefore, although the flux of ions through the fragment channel is greater than through the whole molecule channel, the open fragment channel is much less stable than the open whole molecule channel. In parallel with the reduced stability, the large fluctuations of current through the fragment channel may indicate greater conformational flexibility in the open state of the fragment channel than of the whole molecule channel (Sigworth, 1985). The gating of the fragment ion channel also differs from that of the whole molecule. While the whole molecule ion channel closes at positive voltages, the 18 kD fragment ion channel continues to conduct current, although the magnitude of its conductance is diminished by at least twofold.

It is possible that these differences indicate that portions of the protein outside this ion channel-forming fragment are involved in forming the whole molecule channel. Studies with colicin A truncation mutants (Frenette et al., 1989) and colicin A-E1 fusion proteins (Benedetti et al., 1991) indicate that the colicin A channel-forming domain interacts with the middle third of the protein, the receptor-binding domain. Analogously, portions amino-terminal to the colicin Ia 18 kD fragment may interact with this channel-forming domain and may be necessary to form the whole colicin Ia channel. These portions may confer stability to the open ion channel, perhaps by anchoring it in the membrane, and may also be involved in some aspects of the gating of the whole molecule.

Structurally, the 18 kD fragment of colicin Ia

probably constitutes a stable core of the protein as seen from its protease resistance, and is probably similar to the compact, structural domain of the colicin A channel-forming fragment (Parker et al., 1989; Parker et al., 1990). The colicin A fragment is composed of 10 α-helices arranged in a three-layer bundle, with helices 8 and 9 forming the hydrophobic core. The channel-forming fragment of colicin E1 as determined by NMR is consistent with this structure, except that helices 9 and 10 are slightly rearranged relative to colicin A (Wormald et al., 1990). The channel-forming colicin Ia fragment is slightly shorter than the colicin A fragment but about the same size as the colicin E1 fragment. By sequence comparison, the amino-terminus of the 18 kD fragment corresponds to helix 1 of the colicin A fragment without the first seven residues; its likely carboxy-terminus at residue 626 corresponds to the colicin A fragment without its last three residues. Therefore, the colicin Ia fragment is probably structurally similar to both the colicin A and E1 fragments, although confirmation of this awaits the x-ray crystal structure of colicin Ia (Ghosh, 1992).

The marked differences in functional properties between the 18 kD fragment and the colicin A and E1 fragments then likely arise from small, specific differences in structure arising from differences in sequence. However, it is also possible that although the soluble structures of these fragments may be similar, the membrane-inserted structures could vary considerably from each other. The channel-forming domains of the colicins share little sequence identity, about 25–30%, but have in common sequences that are capable of forming amphipathic α-helices and a hydrophobic stretch of about 35 amino acids. Since their secondary and tertiary structures are likely to be similar but their ion channel properties differ, the channel-forming domains of these colicins provide a useful way to identify sequence elements responsible for specific ion channel properties.

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