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Constraints imposed by protease accessibility on the trans-membrane and surface topography of the colicin E1 ion channel

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Abstract

The surface topography of a 190-residue COOH-terminal colicin E1 channel peptide (NH_2-Met 333–Ile 522-COOH) bound to uniformly sized 0.2-μm liposomes was probed by accessibility of the peptide to proteases in order (1) to determine whether the channel structure contains trans-membrane segments in addition to the four α-helices previously identified and (2) to discriminate between different topographical possibilities for the surface-bound state. An unfolded surface-bound state is indicated by increased trypsin susceptibility of the bound peptide relative to that of the peptide in aqueous solution. The peptide is bound tightly to the membrane surface with $K_d < 10^{-7}$ M. The NH_2-terminal 50 residues of the membrane-bound peptide are unbound or loosely bound as indicated by their accessibility to proteases, in contrast with the COOH-terminal 140 residues, which are almost protease inaccessible. The general protease accessibility of the NH_2-terminal segment Ala 336–Lys 382 excludes any model for the closed channel state that would include trans-membrane helices on the NH_2-terminal side of Lys 382. Lys 381–Lys 382 is a major site for protease cleavage of the surface-bound channel peptide. A site for proteinase K cleavage just upstream of the amphiphilic gating hairpin (K420–K461) implies the presence of a surface-exposed segment in this region. These protease accessibility data indicate that it is unlikely that there are any α-helices on the NH_2-terminal side of the gating hairpin K420–K461 that are inserted into the membrane in the absence of a membrane potential. A model for the topography of an unfolded monomeric surface-bound intermediate of the colicin channel domain, including a trans-membrane hydrophobic helical hairpin and two or three long surface-bound helices, is proposed.

Keywords: colicin; membrane α-helix; membrane protein structure; protein import; voltage-gated channel

The plasmid-encoded colicin E1 molecule (MW = 57,279) is cytotoxic to sensitive cells containing the vitamin B_{12} (*btu*B) receptor and the *tol* gene products for macromolecule translocation across the *Escherichia coli* cell envelope. The cytotoxic action of the colicin is exerted through the formation of a highly conductive ion channel in the cytoplasmic membrane that causes inhibition of active transport and subsequent depletion of ATP and internal K^+ levels. The formation of the channel requires a trans-membrane (negative inside) potential, and colicin E1 behaves electrophysiologically like a voltage-gated channel (Schein et al., 1978; Bullock et al., 1983; Cleveland et al., 1983). Profound structural transitions are known to be involved in the conversion of the water-soluble colicin molecule, or the COOH-terminal channel peptide (Dankert et al., 1982), to the membrane-inserted channel because the structure of the ion channel must involve trans-membrane domains, and the channel domain in its low pH translocation-competent state becomes soluble in nonionic detergent (Merrill et al., 1990). The structural transition of the water-soluble channel peptide, for which the three-dimensional structure of the analogous colicin A channel peptide is known to 2.4 Å resolution (Parker et al., 1989, 1992), probably occurs in vitro through a dynamic or molten globule intermediate (Merrill et al., 1990; van der Goot et al., 1991) and in vivo through a similar state that is imposed by the translocation (*tol*) proteins. The proposed structure of the channel as an apparently

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Abbreviations: DMG, dimethylglutaric acid; FTIR, Fourier transform infrared; LUV, SUV, large, small, unilamellar vesicles; MW, molecular weight; OD, optical density; $\Delta\psi$, trans-membrane potential; PVDF, polyvinylidene difluoride; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; TCA, trichloroacetic acid; YT, yeast extract-tryptone.

monomeric pore (Peterson & Cramer, 1987; Slatin, 1988; Levinthal et al., 1991) includes at least two helical hairpins (four helices) that have been identified (Merrill & Cramer, 1990; Song & Cramer, 1991). These two helical hairpins include (1) a 35–40-residue hydrophobic segment that is believed to insert into the membrane bilayer as a trans-membrane helical hairpin as part of a surface-bound intermediate state in the absence of a membrane potential (Cleveland et al., 1983; Parker et al., 1989; Song et al., 1991), and (2) an amphiphilic 35–40-residue hairpin whose insertion is dependent upon a membrane potential (Merrill & Cramer, 1990). A trans-membrane orientation of the long hydrophobic segment is thermodynamically likely (Engelman & Steitz, 1981) and is implied (1) in vitro by the neutral (Rath et al., 1991) or positive (Goormaghtigh et al., 1991) infrared dichroism of the peptide in oriented membranes, and (2) in vivo by the pattern of site-directed charge-carrying mutations in this segment (Song et al., 1991). The voltage-driven translocation of the long amphiphilic segment into the membrane has been proposed to account for the mechanism of voltage gating (Merrill & Cramer, 1990), and residue His 440 has been implicated as one of the gating charges (Abrams et al., 1991). The present study investigates (1) the topography of other peptide segments of the channel domain that could conceivably form trans-membrane helices in the "open" channel structure, and (2) the surface topography of the membrane-bound "closed" channel intermediate state in the absence of a membrane potential.

Results

As a result of previous studies on the identity of the trans-membrane peptide segments of the colicin E1 ion channel (Merrill & Cramer, 1990; Song et al., 1991), several models of the protein folding pattern of the open channel in the membrane bilayer can be considered (Fig. 1A–F). The underlying assumptions for all the models are (1) the channel is monomeric, based on one-hit cell-killing curves in vivo (Jacob et al., 1952) and a first-order concentration dependence of channel activity in vitro in planar bilayer membranes (Slatin, 1988) and at low peptide concentrations with membrane vesicles (Peterson & Cramer, 1987; Levinthal et al., 1991). (2) The COOH-terminus of the channel is on the cis side of the membrane, based on accessibility to carboxypeptidase of the peptide bound to planar membrane bilayers (Cleveland et al., 1983) or membrane vesicles (Xu et al., 1988). (3) The channel has a predominantly α-helical structure both in solution (Brunden et al., 1984) and in the membrane as inferred from FTIR analysis of secondary structure (Goormaghtigh et al., 1991; Rath et al., 1991; Suga et al., 1991). A significant contribution of β structure was not found in colicin E1 (Rath et al., 1991) or colicin A channel peptide (Goormaghtigh et al., 1991) proteoliposomes, whereas Suga et al. (1991) found an increase in β structure up to 30% of the total peptide when channel peptide was added to liposomes. (4) The channel contains an even number of helices, so that the NH_2-terminus of the channel is also on the cis side of the membrane. The basis for the latter assumption is (1) the cis-side location of the COOH-terminus (Cleveland et al., 1983; Xu et al., 1988), and (2) the trypsin rescue phenomenon (Dankert et al., 1980) that also implies a cis-side location of the NH_2-terminus. In this experiment, trypsin added to the outside of cells, whose energy metabolism has been inhibited by the colicin channel, is able to reverse the deleterious effect of the colicin. Because the channel domain in the colicin molecule is localized to its COOH-terminal end, and trypsin does not affect receptor function in the outer membrane, trypsin must act on an NH_2-terminal segment of the colicin molecule exposed at the cell exterior. Therefore, the colicin channel must span the cytoplasmic membrane an even number of times. The cytotoxicity of colicin E1 linked to Sepharose beads (Lau & Richards, 1976) is also consistent with a trans-envelope arrangement of the colicin molecule.

The assumption of an even number of trans-membrane helices leads to models of four (Fig. 1F) or six (Fig. 1B,D) trans-membrane helices in the open channel. Opening of the channel is associated with the insertion into the membrane bilayer of an amphiphilic helical hairpin, helices H3–H4 in Figure 1A–D or H1–H2 in Figure 1E,F, upon imposition of a trans-negative membrane potential, as shown by differential ($\pm$ membrane potential, $\Delta\psi$) labeling studies (Merrill & Cramer, 1990).

Channel models

Generalized schematic structures of the closed channel in the absence of the membrane potential (Fig. 1A,C,E) depict the molecule as unfolded on the surface of the membrane with four (Fig. 1A,C) or two (Fig. 1E) helices inserted into the bilayer.

Unfolded conformation of surface-bound peptide

A relatively unfolded conformation of the membrane surface-bound intermediate can be seen by the increased sensitivity to trypsin (T, Fig. 2) of the membrane-bound colicin E1 vs. colicin E1 in solution (Fig. 2, lane 3 vs. lane 2) and of the membrane-bound 190-residue COOH-terminal channel peptide, P190 (Fig. 2, lane 5 vs. lane 4). In both colicin and channel peptide, the ability of trypsin to generate an M_r ~14,000 cleavage product is greatly enhanced when the molecule is surface bound. The increased trypsin accessibility/sensitivity of the bound peptide is also observed when the effect of trypsin on a channel peptide–liposome suspension was examined as a function of pH (Fig. 3). The peptide (P190) is not bound at pH 6 (Davidson et al., 1985), and, although trypsin activity is far greater at pH 6 than 4, the proteolysis of the

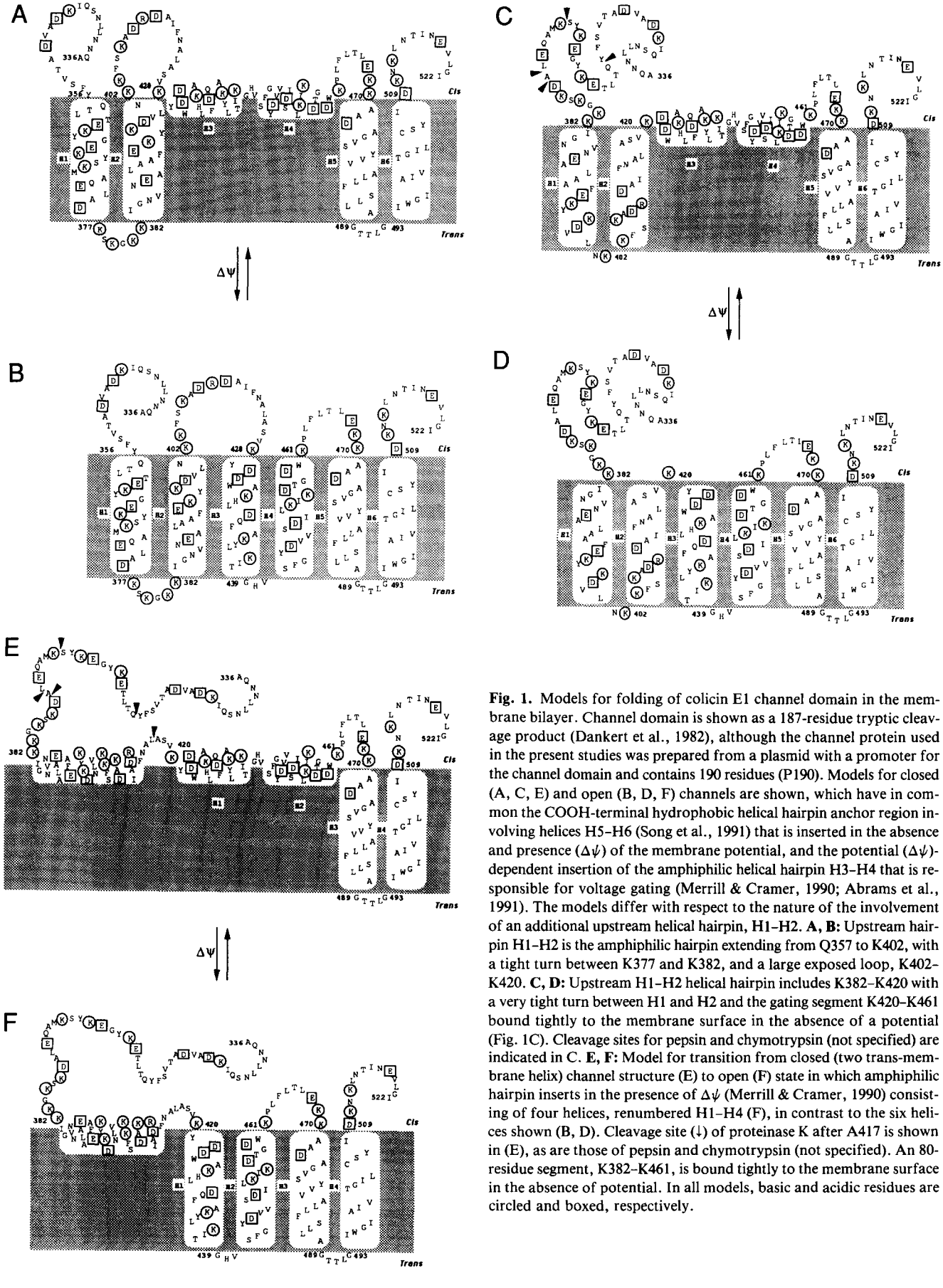

Fig. 1. Models for folding of colicin E1 channel domain in the membrane bilayer. Channel domain is shown as a 187-residue tryptic cleavage product (Dankert et al., 1982), although the channel protein used in the present studies was prepared from a plasmid with a promoter for the channel domain and contains 190 residues (P190). Models for closed (A, C, E) and open (B, D, F) channels are shown, which have in common the COOH-terminal hydrophobic helical hairpin anchor region involving helices H5–H6 (Song et al., 1991) that is inserted in the absence and presence ($\Delta\psi$) of the membrane potential, and the potential ($\Delta\psi$)-dependent insertion of the amphiphilic helical hairpin H3–H4 that is responsible for voltage gating (Merrill & Cramer, 1990; Abrams et al., 1991). The models differ with respect to the nature of the involvement of an additional upstream helical hairpin, H1–H2. **A, B:** Upstream hairpin H1–H2 is the amphiphilic hairpin extending from Q357 to K402, with a tight turn between K377 and K382, and a large exposed loop, K402–K420. **C, D:** Upstream H1–H2 helical hairpin includes K382–K420 with a very tight turn between H1 and H2 and the gating segment K420–K461 bound tightly to the membrane surface in the absence of a potential (Fig. 1C). Cleavage sites for pepsin and chymotrypsin (not specified) are indicated in C. **E, F:** Model for transition from closed (two trans-membrane helix) channel structure (E) to open (F) state in which amphiphilic hairpin inserts in the presence of $\Delta\psi$ (Merrill & Cramer, 1990) consisting of four helices, renumbered H1–H4 (F), in contrast to the six helices shown (B, D). Cleavage site (↓) of proteinase K after A417 is shown in (E), as are those of pepsin and chymotrypsin (not specified). An 80-residue segment, K382–K461, is bound tightly to the membrane surface in the absence of potential. In all models, basic and acidic residues are circled and boxed, respectively.

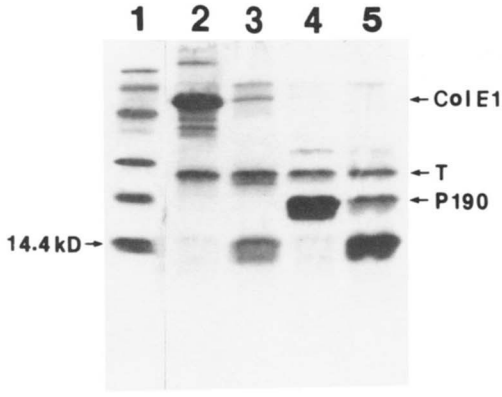

Fig. 2. Increase in accessibility to trypsin of membrane-bound colicin or channel-forming peptide. Lane 1, molecular weight markers: phosphorylase B, 97.4 kDa; bovine serum albumin, 66.2 kDa; ovalbumin, 45 kDa; carbonic anhydrase, 31 kDa; soybean trypsin inhibitor, 21.5 kDa; lysozyme, 14.4 kDa. Lanes 2 and 3, trypsin cleavage of colicin E1 at pH 4 in the absence and presence of liposomes, respectively. Lanes 4 and 5, trypsin cleavage of colicin E1 channel-forming peptide at pH 4 in the absence and presence of liposomes, respectively. Proteolysis reaction, 35 °C, 1 h. Protease/protein ratio was 1/5 (w/w). The presence of trypsin (T) is seen in the gel.

bound peptide at pH 4 is far more extensive (lane 1 vs. lane 3). A conformational change of the colicin E1 channel when bound to phosphatidylcholine membranes at pH 4 was inferred from the insensitivity of its channel properties to further changes in pH (Bullock, 1992). An unfolded state of the colicin A channel peptide on the surface of the membrane has also been inferred from analysis of changes in fluorescence-resonance energy transfer (Lakey et al., 1991).

The affinity of the channel peptide for the membrane surface was determined using labeled peptide and found to be $<10^{-7}$ M (data not shown), indicating very tight binding and a high affinity of the channel peptide for the membrane. This figure for the binding constant is a conservative one. It includes a correction for peptide that sediments in the absence of liposomes and assumes that all such peptide remains free and sediments without binding when the latter are present. It is likely that some of this peptide actually binds and that the K_d is smaller than 7×10^{-7} M.

It is important to note that the recovery from liposomes of colicin and channel peptide (P190), as well as its major proteolytic cleavage product, is quantitative (Fig. 4). A comparison of lanes 5 and 6 with lanes 3 and 4 (Fig. 4) shows that there is no obvious loss of colicin or peptide upon its extraction from liposomes. Lanes 7 and 8 (Fig. 4) show that an M_r 14,000 peptide is the dominant or exclusive product of trypsin proteolysis of liposome-bound colicin and peptide. The proteolysis of bound channel peptide (P190) is less complete than that of colicin.

Identity of trans-membrane helices

Four segments in the colicin COOH-terminal domain trans-membrane helices have been identified as probable trans-membrane helices (Merrill & Cramer, 1990; Song et al., 1991). These four helices are organized as two α-helical hairpins, the hydrophobic anchor K470–D509 and the amphiphilic hairpin, K420–K461. The former is believed to become inserted into the membrane as part of the process of binding of the soluble channel peptide to the membrane surface. No further insertion of this hydrophobic "anchor" peptide was seen upon imposition of a membrane potential, which caused labeling changes read-

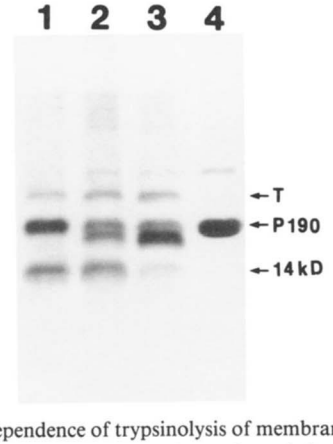

Fig. 3. The pH dependence of trypsinolysis of membrane-bound channel peptide. Lanes 1–3, 10 μg of membrane-bound channel peptide digested by trypsin at pH 4, 5, and 6, respectively. Lane 4, 10 μg of membrane-bound channel peptide recovered from liposomes. Proteolysis, 35 °C, 1 h.

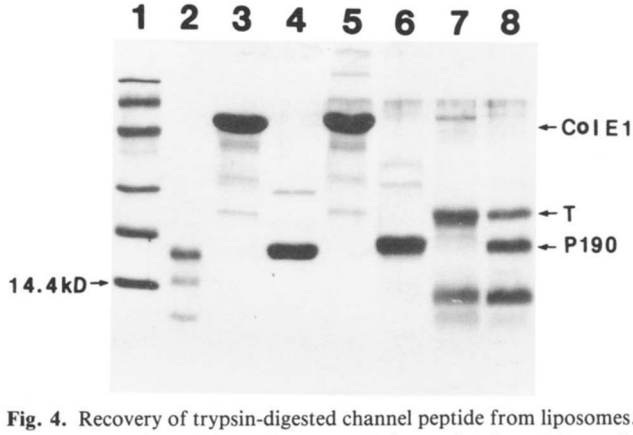

Fig. 4. Recovery of trypsin-digested channel peptide from liposomes. Lane 1, molecular weight markers. Lane 2, low molecular weight markers: myoglobin, 16.9 kDa; myoglobin I+II fragment, 14.4 kDa; and myoglobin I fragment, 6.2 kDa. Lanes 3 and 4, 10 μg of colicin and channel peptide loaded directly for SDS-PAGE, respectively. Lanes 5 and 6, 10 μg of colicin and channel peptide recovered from liposomes after the same treatment as the digested products, respectively. Lanes 7 and 8, 10 μg of colicin and channel peptide recovered from liposomes after trypsin digestion, respectively. Proteolysis reaction, 35 °C, 1 h.

ily interpreted in terms of insertion of the amphiphilic gating helical hairpin, K420–K461, into the membrane (Merrill & Cramer, 1990). No other segments of the channel domain were seen to insert into the membrane in the presence of a potential. However, two other upstream regions, Q357–D376 and K383–N401, are very amphiphilic and might be imagined to be inserted into the membrane in the absence of a potential (Fig. 1A) as an additional helical hairpin, making a six-helix (H1–H6) open channel in the presence of a membrane potential (Fig. 1B). The hydrophobic moments, μ_α (Eisenberg et al., 1984) of the peptide segments, Q357–D376, I383–N401, S418–L435, and N440–T457, using the Eisenberg consensus database for residue hydrophobicity, are 0.42, 0.25 (0.33 for N385–K403), 0.42, and 0.35, respectively, calculated for an α-helical periodicity of 100°. If Q357–D376 and I383–N401 constitute a membrane-spanning helical hairpin in the open channel, these segments should be inaccessible to protease added to membrane vesicles containing bound colicin or channel peptide. However, several proteases cleave colicin or channel peptide bound to the membrane vesicles. Trypsin cleaves liposome-bound colicin (Figs. 2–4) to an M_r ~14–15-kDa fragment. This peptide has a unique, although ragged NH_2-terminus, NH_2-Lys 382 or NH_2-Ile 383, determined by sequencing (Table 1; cf. Xu et al., 1988). Other sites in the putative trans-membrane helices H1–H2 of the channel model shown in Figure 1A were accessible to different proteases (Fig. 5; Table 1B,C): (1) chymotrypsin was found to cleave before Ser 368 and Ala 375 (Table 1B), resulting in the somewhat broad band containing two peptide fragments seen in lane 5 of Figure 5A at M_r values >14,000–15,000; (2) pepsin generates a band just below the parental channel peptide (P190) (lane 6, Fig. 5A) resulting from cleavage before Tyr 356 and Leu 374 (Table 1C). (3) Other proteases, thermolysin (Fig. 5B, lane 2), V8 protease (Fig. 5B, lane 3), and proteinase K (Fig. 5B, lane 1) also caused cleavage of the P190 channel peptide in this region (Table 1D–F).

Thus, in contrast to the model shown in Figure 1A, it is clear that the segment Tyr 356-Lys 382 does not span the bilayer but is only loosely bound to the membrane surface or not even bound.

The region of the channel domain on the NH_2-terminal side of the hydrophobic anchor region K470–K510 that either spans the bilayer (Fig. 1C), or is bound tightly to the membrane surface (Fig. 1E) in the absence of a potential, is a 80–90-residue segment extending from approximately Lys 382 to Lys 461 or Lys 470 (Fig. 6). In spite of the presence of 16 Lys, 1 Arg, 10 Asp, and 3 Glu residues in the segment, it contains no apparent sites for trypsin or V8 protease, nor any for thermolysin, chymotrypsin, and pepsin. This is consistent with close proximity of the peptide to the membrane surface and the high degree of overall liposome binding affinity ($K_d < 10^{-7}$ M) possessed by the channel peptide.

It was possible to find one site of protease accessibility in the surface-bound channel peptide in the segment Lys 382–Lys 470. Proteinase K cleaves between Leu 416 and Ala 417 (Fig. 5B, lane 1; Table 1F), showing that there is an exposed segment or loop close to the NH_2-terminal end of the amphiphilic helical hairpin, K420–K461 (Fig. 6). The exposure of Leu 416-Ala 417 to the cis-side aqueous phase implies that helix H2 is too short to span the bilayer and does not occur as shown in Figure 1C,D. Alternatively, the above data may be described by a model in which trans-membrane helices H1–H2 of the six-helix model are tightly bound to the membrane surface and end in an exposed loop containing residues Leu 416–Ala 417 (Fig. 1E). Upon imposition of a membrane potential, the amphiphilic helical hairpin (K420–K461) would insert into the bilayer as demonstrated (Merrill & Cramer, 1990), creating a four-helix channel,

Table 1. *NH_2-terminal sequence of cleaved membrane-bound colicin E1 channel peptide after incubation with protease*

Protease	Reference	Sequence[a]	NH_2-terminal residue of cleavage product
A. Trypsin	Xu et al. (1988)	I/K, G/I, N/G, V/N, N/V, E/N, A/E, L/A, A/L, A/A	K382, I383
B. Chymotrypsin	This study (Fig. 5A)	S/A, K/D, M/K, A/S, Q/K, E/G	S368, A375
C. Pepsin	This study (Fig. 5A)	Y/L, Q/A, T/D, L/K, T/S, E/K	Y356, L374
D. V8 protease	This study (Fig. 5B)		Between I336 and K381[b]
E. Thermolysin	This study (Fig. 5B)		Between I336 and K381[b]
F. Proteinase K	This study (Fig. 5B)	A, S, V, K, Y, D	A417, and between I336 and K382[b]

[a] Microsequencing procedure described above in Materials and methods. For trypsin, chymotrypsin, and pepsin, two amino acids were found in each cycle. In Xu et al. (1988), 25 cycles were carried out that matched the amino acid sequence predicted from colicin E1 gene (Yamada et al., 1982), although 10 cycles were presented. In this study, six cycles were determined as shown.

[b] Proteinase K also cleaves the membrane-bound colicin E1 channel-forming peptide at a residue between I336 and K381. Because this fragment had approximately the same molecular weight as that generated by chymotrypsin and pepsin, it was not sequenced. For the same reason, the two fragments generated by V8 protease and thermolysin, respectively, were not sequenced.

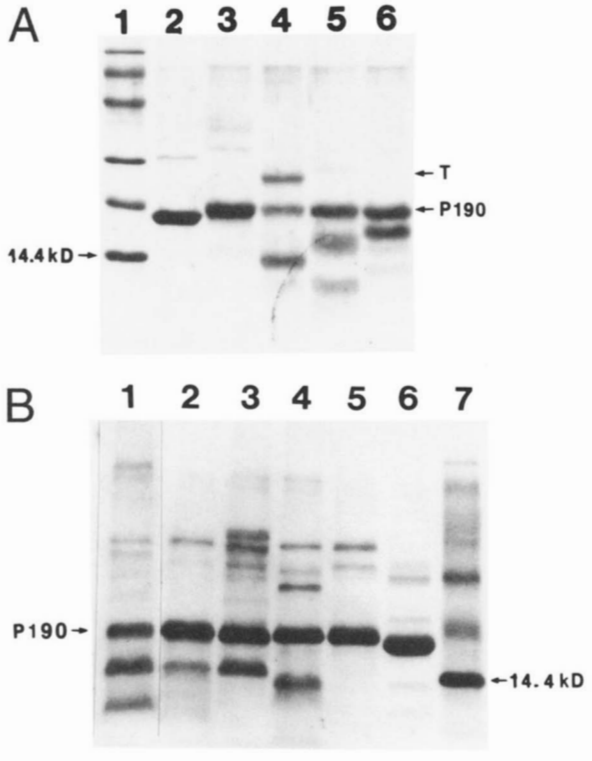

Fig. 5. Effect of other proteases on membrane-bound colicin E1 channel peptide, P190. **A:** Chymotrypsin and pepsin digestion of membrane-bound channel peptide at pH 4. Lane 1, molecular weight markers. Lane 2, 10 μg of channel peptide loaded directly for SDS-PAGE. Lane 3, 10 μg of channel peptide recovered from lipid. Lane 4, trypsin cleavage of 10 μg of membrane-bound channel peptide for 1 h. Lanes 5 and 6, chymotrypsin and pepsin cleavage of 10 μg of membrane-bound channel peptide for 1 h, respectively. **B:** Proteinase K, thermolysin, V8 protease digestion of membrane-bound channel peptide at pH 4. Lane 1, proteinase K cleavage of membrane-bound channel peptide for 10 min. Lanes 2 and 3, thermolysin and V8 protease cleavage of membrane-bound channel peptide, respectively. Lane 4, trypsin cleavage of membrane-bound channel peptide. Lane 5, colicin and channel peptide recovered from liposome after the same treatment as the digested products. Lane 6, channel-forming peptide loaded directly. Lane 7, molecular weight markers. Ten micrograms of channel peptide were added to all lanes; cleavage time, 1 h, for all proteases except proteinase K in lane 1.

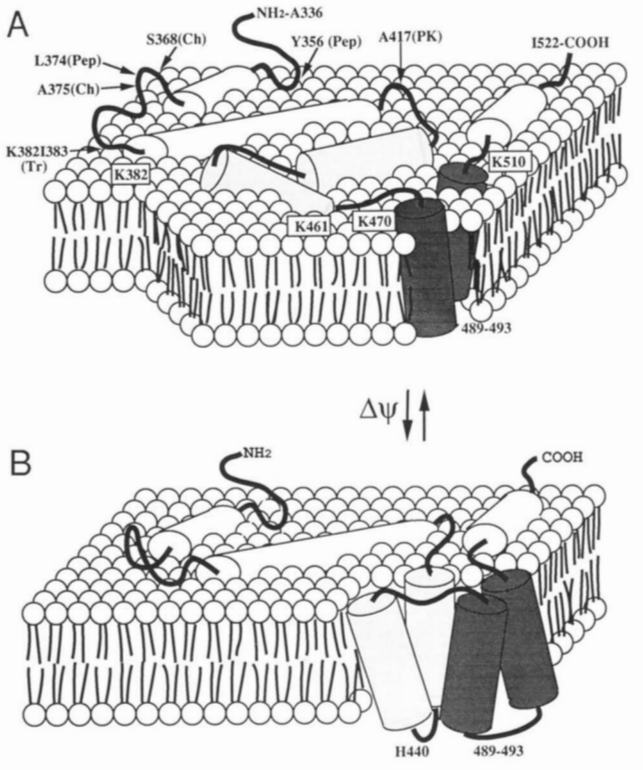

Fig. 6. Model for surface-bound intermediate (**A**) and for a four-helix model of the colicin E1 channel in the membrane in the presence of a membrane potential, $\Delta\psi$ (**B**), inferred from photoaffinity labeling (Merrill & Cramer, 1990), mutagenesis of hydrophobic (dark, shaded) segment (Song et al., 1991), protease accessibility data presented in this work, and discussion of the role of this segment in insertion presented previously for colicin E1 by Cleveland et al. (1983) and for colicin A by Parker et al. (1989, 1992). Protease cleavage sites that are documented in Table 1 are shown. The initial surface-bound state that results from interaction of soluble channel peptide with the membrane bilayer containing anionic lipid is unfolded on the surface and bound tightly ($K_d < 10^{-7}$ M) to it. Tight binding is a consequence of both electrostatic interaction of positively charged peptide with negative membrane surface and insertion of the hydrophobic hairpin. In the presence of a membrane potential ($\Delta\psi$), the amphiphilic helical hairpin, K420–K461 (lightly shaded helical hairpin), inserts into the bilayer, thus gating the channel (Merrill & Cramer, 1990; Abrams et al., 1991).

with K420–K461 defining a trans-membrane helical hairpin with helices H1–H2 (Fig. 1F). Another possibility that will be discussed below is that the shortened segment K382–A415 might undergo a $\Delta\psi$-dependent insertion as a β-structure hairpin.

Discussion

Number of trans-membrane helices

The many accessible protease sites between NH_2-Met 333 and Lys 381 of the membrane-bound P190 channel peptide imply that this region cannot be inserted into the membrane in the absence of a membrane potential. The apparent lack of association of this region with the membrane surface indicates that (1) it is not inserted into the membrane in the absence of a membrane potential; in addition, there was no proteolysis of inserted peptide at pH 4 by an excess of encapsulated trypsin, under conditions where cleavage of apocytochrome *c* at pH 7 could be demonstrated (data not shown). (2) The loose binding of the M333–K381 segment indicates that it is not positioned to be inserted into the membrane bilayer by imposition of a potential, and the insertion of this region upon imposition of a $\Delta\psi$ was not observed (Merrill & Cramer, 1990). Therefore, models 1A and B for the colicin channel are excluded.

Labeling studies showed no evidence for potential dependent insertion of the segment on the NH_2-terminal side of Lys 420 (Merrill & Cramer, 1990). Therefore, the model of Figure 1C,D was considered, with a hairpin extending from Lys 382 to Lys 420 inserted into the bilayer in the absence of a potential. These models also describe a six-helix structure in the open channel state. The proposed helices H1–H2 shown in Figure 1C,D would constitute a tight helical hairpin structure, because a typical membrane-spanning α-helix contains approximately 20 residues, and at least 3–5 residues should be allowed for a turn. The existence of a proteinase K site between Leu 416–Ala 417 would leave no more than 34 residues available for such a hairpin, arguing against the existence of helix H2 in the absence of a potential. It can also be argued that it is unlikely that the Lys 382–Lys 420 could insert spontaneously into the membrane bilayer in a monomeric channel structure in the absence of a $\Delta\psi$ because it contains 11 charged residues, 6 basic (Lys 395, Lys 397, Lys 402, Lys 403, Lys 406, Arg 409) and 5 acidic (Glu 388, Glu 394, Asp 398, Asp 408, Asp 410). The channel competence of a 136-residue COOH-terminal peptide of colicin A (Baty et al., 1990) would also argue that neither a complete helix H1 nor H2, as drawn in Figure 1A,B or C,D, is necessary for a functional channel, although such upstream segments do influence its gating properties (see below, Role of NH_2-terminal surface-bound segments in channel gating).

These data and the experiment of Baty et al. (1990) imply that a functional channel can consist of only the four helices shown in Figure 1E,F. A graphics model of the four-helix, H1–H4, channel shown schematically in Figure 1F has been constructed, with appropriate van der Waals and H-bond distances (Cramer et al., 1992). Some features of this model are (1) the 18 charged residues in the four helices, 9 Lys and 9 Asp, all point away from the lipid phase except for Lys 455; (2) the 11 aromatic residues all face outwardly into the lipid except for Trp 460 near the cis-side aqueous interface and Phe 484, which is in a hydrophobic pocket between H3 and H4; (3) the effective diameter of the channel lumen cannot be accurately determined, because it depends on side-chain conformation. However, it could be 8–10 Å with small allowed changes of side-chain torsion angles.

Problems with the four-helix model are (1) there is no precedent in models of native channels (Stephan and Agnew, 1991), although a four-helix model has been proposed from studies on voltage-gated Ca^{2+} channels using synthetic peptides (Grove et al., 1991). It has been proposed that the paucity of helices in the colicin channel could be solved if the colicin channel is an oligomer, specifically a trimer (Parker et al., 1992). A difficulty with this proposal is that the existing data all indicate that the channel formation process is first order in monomeric colicin or peptide concentration (Cramer et al., 1990). The simplest, most convincing first-order experiment is that of cytotoxicity (Jacob et al., 1952) for which it is impossible to fit the data, in an experiment that has been done innumerable times in many laboratories, by a three-hit process. (2) It is not clear that a four-helix model can accommodate a channel lumen of at least 8 Å (Raymond et al., 1985; Bullock et al., 1992), although this depends on the exact conformation and torsion angles of the side chains in the lumen space of the channel (Cramer et al., 1992).

Because of these problems, one might also logically argue for the presence of a hairpin in the β-sheet conformation spanning the bilayer from Lys 382 to Ala 415 that could insert in the presence of $\Delta\psi$ along with the helical hairpin. Although two FTIR studies of channel peptide–proteoliposomes did not detect any β structure, a third study in which the channel peptide was added to preformed liposomes reported a content of 30% β structure. The β structure content that would be predicted with approximately 25 residues in the Lys 382–Leu 416 segment in this conformation in the 178-residue thermolytic channel peptide would be ca. 14%. There is a precedent for such a hairpin in the channel lining of the Na^+, Ca^{2+} channels (Hartmann et al., 1991; Yellen et al., 1991). The arguments against such voltage-gated β structures are (1) the competence of a short peptide in the analogous colicin A peptide missing most of the Lys 382–Ala 415 region (Baty et al., 1990); (2) labeling evidence indicating that the region K382–K420 does not undergo $\Delta\psi$-dependent insertion into the bilayer (Merrill & Cramer, 1990); (3) the thermodynamic difficulties of inserting a β hairpin without an oligomeric structure and of converting a probable surface-bound helical structure (μ_α of N385–K403 = 0.33) to a β structure.

Thus, it is difficult to find evidence for trans-membrane segments other than the four helices between K420 and K510. This structure appears bare, particularly as a monomer, when compared to those proposed for eukaryotic Na^+, Ca^{2+}, and K^+ channels. However, it is important to remember that the function of the colicin channel may be far less precise and unregulated compared to eukaryotic channels that have a role in cell metabolism. On the other hand, a caveat to the present data is that they were obtained in an in vitro liposome system. It is possible that segments upstream of K420 can be inserted in vivo by appropriate translocation "helper" proteins.

The conclusion of Liu et al. (1986) that a colicin E1 channel peptide starting at D429 retained channel activity might appear to eliminate the need for helix H1 in the four-helix model (Fig. 1F). However, the channel activity measured by Liu et al. (1988) is assumed here to somehow be artifactual (e.g., arising from background channel activity of a hydrophobic peptide) because those involved in the inference made by Liu et al. (1986) do not take it into account in their recent study on the mechanism of gating of the colicin channel (Abrams et al., 1991). The four-helix model presented in that study includes helix H1 of Figures 1F and 6.

Conformation change upon binding and insertion

The existence of a conformational or structural change of colicin E1 upon binding to the membrane is shown in the present work by increased protease sensitivity of the bound peptide (Fig. 2) and has been shown for the colicin A peptide by changes in fluorescence energy transfer (Lakey et al., 1991). The need for such a structural change in the case of the channel-forming colicins was recently discussed in studies on annexin-V, a human placental anticoagulant protein that binds phospholipids with high affinity in a Ca^{2+}-dependent manner and forms Ca^{2+}-specific voltage-gated channels (Brisson et al., 1991). Annexin crystallized from aqueous solution corresponds closely in shape with the molecule studied by electron diffraction analysis in its membrane-associated form. It was proposed that, in contrast to the model proposed for channel-forming colicins, the formation of the annexin channel is unlikely to involve integration into the membrane. It is possible that channel formation by annexin is fundamentally different than what is inferred for the open state of the colicin and, moreover, all other channels discussed in the literature. A more conservative, simpler explanation is that (1) only the closed state of the annexin channel was observed in the structural studies, and (2) the closed state does not involve any membrane-inserted segment, but the binding affinity of annexin gives it the possibility of integrating into the bilayer upon imposition of a membrane potential.

Tight binding of channel peptide to the membrane surface—Significance for insertion of hydrophobic helical domains

The binding of the colicin channel peptide to the membrane surface at pH 4.0 can be compared with the binding of apocytochrome *c* at neutral pH because both proteins are highly cationic under these conditions. By such a comparison, the binding of the channel peptide to the LUV used in the present study with $K_d < 10^{-7}$ M can be considered high affinity because the K_d, for high affinity apocytochrome *c* binding to LUV is approximately 20 μM (Rietveld et al., 1986). The affinity ($K_d < 1$ μM) is greater for apocytochrome *c* binding to SUV (Berkhout et al., 1987). We would propose that tight binding arising from electrostatic attraction of the cationic colicin peptide for the negative membrane surface is a pre- or corequisite for insertion of its hydrophobic hairpin region into the membrane bilayer. The reason for this assertion is that the labeling of the inserted segment(s) of the colicin channel peptide by hydrophobic photoaffinity labeling compounds decreased greatly when the pH of the liposome suspension, to which channel peptide had been added at pH 4.0, was increased to 6.0 (Xu et al., 1988). The major effect of this pH shift is expected to be a decrease in the electrostatic component of the binding energy. The shift to pH 6 was not expected to cause release of the inserted hydrophobic segment from the membrane. The anchoring of the colicin E1 channel peptide probably results from (1) tight electrostatic binding to the surface of amphiphilic helical segments and (2) concomitant insertion of the hydrophobic domain (Fig. 6A).

Topography of surface-bound peptide

An important perspective is that the structure of the colicin channel is likely to be very flexible. The inaccessibility to protease of the 141-residue segment Lys 382–Ile 522-COOH implies that in the absence of membrane potential it is bound tightly to the membrane surface, except for the hydrophobic helical hairpin that is believed to be inserted into the membrane bilayer. The one small exposed region is around Leu 416–Ala 417. The large α-helical amphipathic moment (μ_α) of segments Lys 385–Lys 403 and Lys 420–Lys 461 implies that the surface-bound peptide segments are bound mostly as surface-seeking amphipathic helices (Fig. 6). A consequence of this surface conformation is that α-helical conformation of the colicin E1 (Rath et al., 1991; Suga et al., 1991) and A (Goormaghtigh et al., 1991) channel peptide incorporated into or bound to liposome membranes, indicated by the FTIR determination, may have been heavily influenced by the surface segments.

The structure of the surface-bound intermediate of the colicin channel domain can be described as an "unwrapped" or "umbrella" model. The latter term has been used to describe the nature of the surface state predicted for the analogous colicin A channel (Parker et al., 1989; Lakey et al., 1991). A difference between the unwrapped model described in the present work (Fig. 6A,B) and the umbrella model is that the latter makes the specific prediction that the identity of α-helical segments is conserved in the transition from the soluble to the membrane-bound and membrane-inserted states.

Role of NH_2-terminal surface-bound segments in channel gating

At least three sets of experiments indicate that segments upstream (i.e., on the NH_2-terminal side) of the colicin channel membrane-spanning helices, defined in this and other (Merrill et al., 1990; Abrams et al., 1991; Song et al., 1991) studies, significantly influence and modulate the gating properties and function of the channel. These experiments are (1) the in vivo "trypsin rescue" experiment (Dankert et al., 1980) discussed above; (2) the 5–10-fold faster channel gating "off-rate" of a 136-residue COOH-terminal channel peptide (NH_2-N387) compared to that of the 178-residue thermolytic peptide (Raymond et al., 1986); and (3) pepsin added to the cis, but not the trans, side of closed channels in membrane bilayers inactivated channel activity (Slatin et al., 1986). This result is topo-

graphically consistent with the pepsin accessibility data of the present study, but also implies that an upstream peptide-accessible segment is involved in channel activity. It is also possible that the high concentrations of pepsin used in that study, in contrast to the single-hit protease action shown in the present work, caused multihit effects and cleavage of the peptide at additional downstream site(s). Another finding of Slatin et al. (1986) that trans-added pepsin inactivated open, but not closed, channels could not be tested in the present work because of the impossibility of carrying out such experiments with liposomes. The latter bilayer experiment bears on dynamic aspects of channel formation, and on the existence of channel substrates (Bullock, 1992; Cohen et al., 1992) in addition to the simple two-state open-closed models depicted in Figures 1E,F and 6A,B.

Materials and methods

Isolation of colicin E1 and channel peptide

The *lexA*$^-$ DM1187 strain was used for overexpression of the *cea* gene (Song et al., 1991), instead of using mitomycin C to induce overproduction of colicin E1. DM1187/pSKE1(−) cells were inoculated into 2× YT medium (1 L), grown overnight, sedimented, and resuspended in 50 mM sodium borate buffer, 2 mM EDTA, pH 9.5. The resuspended cells were broken with a French press and the lysates sedimented at 10,000 × *g* for 15 min. Clear lysates were loaded on a CM-BioGel cation-exchange column (2.5 cm × 30 cm). The column was washed with several column volumes of the buffer until the OD_{280} of the eluate was lower than 0.01. The colicin was eluted with the same buffer containing 0.3 M NaCl. The purity of the colicin was routinely examined on an SDS-PAGE Phast gel system (Pharmacia). The optical density at 280 nm was measured to determine the concentration using an extinction coefficient of 0.74/mg/mL (Schwartz & Helinski, 1971). Cytotoxicity was measured immediately after purification or with samples that had been frozen at −70 °C, as previously described (Song et al., 1991).

To avoid the disadvantages of using protease cleavage of the colicin E1 molecule to prepare the channel-forming peptide, a plasmid ((pSKHY)(−)) that could directly express the COOH-terminal channel-forming domain of colicin E1 was constructed by deleting the gene encoding the NH_2-terminal fragment of colicin from the pSKE1(−) plasmid that expresses colicin E1 (Song et al., 1991). The resulting 190-residue peptide contained three more residues (MW = 21,046) than the tryptic fragment (MW = 20,684) originally used to define the colicin E1 channel domain (Dankert et al., 1982). The first five residues of the P190 peptide are M-E-T-A-E (cf., wild-type residues 333–337 in colicin E1 molecule are LKKAQ). The overexpression and purification of the channel-forming peptide were slightly different from that of colicin, in that the buffer used to lyse cells and to wash the CM-BioGel column was 50 mM NaOAc buffer at pH 5.3. In vitro channel activities of the peptide were assayed by measuring the rate of Cl^--efflux from Cl^--loaded liposomes with a chloride-specific electrode (Peterson & Cramer, 1987).

Preparation of homogeneous liposomes

LUV were prepared according to Peterson and Cramer (1987) from soybean asolectin (Associated Concentrates, Woodside, New York) or purified by the method of Kagawa and Racker (1971). Forty milligrams of asolectin in 400 μL of chloroform (100 mg/mL stock) was evenly deposited on the wall of a test tube using a stream of N_2 gas, placed under vacuum for at least 1 h, and rehydrated with 2 mL of 0.1 M KCl, 10 mM DMG, 1 mM $CaCl_2$, at pH 5.0. The suspension was sonicated to clarity for 15–30 min under N_2 gas in a bath type sonicator and subjected to two freeze–thaw cycles in a dry ice/ethanol bath. The LUV were then forced 8–10 times by N_2 gas pressure through a double layer of a 0.2-μm filter (Millipore Corporation, Bedford, Massachusetts) fixed on a homemade extruder that was found to generate unilamellar vesicles distributed in size about a mean diameter of 170 nm and a half-width of 40 nm.

Proteolysis of membrane-bound colicin and channel peptide

Eighty microliters of vesicles, 40 μL of 0.5 M NaOAc buffer, pH 4.0, were diluted with distilled H_2O so that after addition of 10 μg of either colicin or channel-forming peptide the final volume would be 200 μL. After the protein–vesicle suspension was incubated at room temperature for at least 40 min to ensure maximum binding, 2 μg of protease freshly dissolved in 0.1 M NaOAc buffer at pH 4.0 was added. The suspension was then incubated at 35 °C for 10 min to 1 h, depending on the protease, as specified in the figure legend. Digestion of the colicin or peptide was terminated by addition of the solutions for extraction of the digested products as follows.

Extraction of peptide or protein from liposomes and microsequencing analysis of digested products

After digestion, 1 volume of chloroform/methanol (2:1) and $\frac{1}{3}$ volume of 15% TCA were added to the reaction medium. The tubes were vortexed for 30 s and incubated on ice for 30 min. The mixture was separated into two phases, the upper aqueous and the lower organic, with the digested protein pellet floating between the two by centrifugation (10^4 rpm, 10 min), in a microcentrifuge. The protein pellet was isolated by carefully removing the two phases with a syringe, washed twice with 1 mL ether, and dried under vacuum to remove residual TCA. The digested products were then solubilized with 10 μL of sample buffer for separation on SDS-PAGE.

The extracted proteolytic fragments were resolved on a Bio-Rad mini-slab-gel SDS-PAGE apparatus (ca. 10 μg/lane, 200 mV, 45 min). Protein bands were transferred electrophoretically (100 mA, 1 h), using either the Hoefer Semi-Dry transfer unit TE70 or the Bio-Rad mini-trans-blot electrophoretic transfer cell onto a PVDF membrane (ICN). After staining with Coomassie blue and destaining, protein bands of interest were excised and analyzed on an Applied Biosystems Model 470A protein sequencer fitted with a reaction cartridge specially designed for PVDF-bound samples, at the Laboratory for Macromolecular Structure of Purdue University.

Measurement of binding constant of channel peptide for surface of membrane vesicles

Extruded asolectin LUV (equivalent to 29.5 nmol Pi) were incubated with 0–20 μg of [^{3}H](Leu)-channel peptide in a total volume of 180 μL. After 30 min incubation at 30 °C in 50 mM NaOAc buffer, pH 4.0, the incubation mixture was centrifuged (130,000 × *g*, 15 min, Airfuge) to separate bound and free channel peptide. One hundred sixty microliters of the supernatant were removed for scintillation counting to determine peptide concentration. Alternatively, the incubation suspension was mixed with 15 μL of 1 M $CaCl_2$ for 15 min on ice and centrifuged at 10,000 × *g* for 10 min for complete precipitation of the vesicles and bound peptide. Analysis of the binding data was according to a binding model $P + L_N = PL_N$ (Hille et al., 1981), where P and L are the free concentrations of peptide and lipid, respectively, and N is the number of lipid molecules that constitute one binding site, with a dissociation constant equal to $[P][L_N]/[PL_N]$.

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