Mapping a Membrane-Associated Conformation of Colicin Ia†

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ABSTRACT: Channel-forming colicins exist in at least two different membrane-associated conformations: a voltage-independent closed-channel state and a voltage-dependent open-channel state. In a voltage-independent membrane-associated conformation, we find that two major regions of colicin Ia are protected from pepsin proteolysis after association with negatively charged membranes. In contrast, colicin Ia is rapidly and completely proteolyzed in the absence of membranes. The major protected region includes an electrophysiologically defined C-terminal channel-forming domain as well as 96 residues upstream of this region. Approximately 100 residues spanning Ala79–~Arg189 within the N-terminal domain are protected as well. The first N-terminal 76 residues of colicin Ia and a large region which includes much of the putative central receptor-binding domain are not protected from proteolysis. Both N- and C- termini of protected peptides have been identified using a combination of gel electrophoresis, N-terminal sequencing, and mass spectrometry, thereby defining specific residues that are located on the outside of the lipid bilayer. These data suggest a role for regions other than the electrophysiologically defined C-terminal channel-forming domain in membrane insertion and channel formation.

Many soluble toxin molecules spontaneously insert into the lipid bilayers of their target cells. Examples include toxins from the human pathogens *Corynebacterium diphtheriae*, *Clostridium tetani*, and *Vibrio cholerae* (Donovan et al., 1981; Holmgren, 1981; Bouquet & Duflot, 1982) as well as the channel-forming colicins, bacteriocins that are synthesized in and secreted by *Escherichia coli*, and which are lethal to other bacteria (Konisky, 1982). These colicins have been widely used in studies aimed at understanding how water-soluble proteins spontaneously insert into membranes.

The channel-forming colicins, which include colicins A, E1, Ia, Ib, K, and N, form lethal ion channels in sensitive target *E. coli* bacteria (Konisky, 1982). After binding to specific bacterial outer membrane receptors, these colicins translocate across the outer membrane and periplasmic space and then insert into and form channels within the inner plasma membrane. The channels deplete the target cells of their electrochemical gradient and cause cell death.

In vitro, colicins A, E1, and Ia form relatively nonselective, voltage-gated ion channels in planar lipid bilayers upon application of a trans-negative voltage (cis is defined as the side of the bilayer to which protein is added, while trans is defined as the opposite side) (Schein et al., 1978). These colicins also associate with membranes in the absence of a transmembrane voltage, binding to and releasing the contents of negatively charged liposomes at acidic pH (Pattus et al., 1983; Davidson et al., 1985; Kayalar & Duzgunes, 1986; Mel & Stroud, 1993). Proteolytic cleavage studies of colicin E1 in planar lipid bilayers (Raymond et al., 1986; Slatin et al., 1986) as well as labeling studies of colicin E1 in the presence and absence of a voltage (Merrill & Cramer, 1990) indicate that the structures of the voltage-dependent and voltage-independent membrane-associated forms differ, however.

Proteolysis and deletion analysis studies of colicins A and E1 reveal that separate domains are responsible for each of the three functions: receptor binding, translocation across the outer membrane, and channel-formation (Ohno-Iwashita & Imahori, 1982; Martinez et al., 1983; Brunden et al., 1984; Cavard et al., 1986; Liu et al., 1986; Baty et al., 1988). Since there is a shared function and 25–30% sequence identity among colicins A, E1, and Ia, it is likely that colicin Ia also contains three domains with similar functions. In fact, it has recently been shown that a C-terminal domain of colicin Ia beginning at Asp451 forms voltage-dependent ion-conducting channels in planar lipid bilayers (Ghosh et al., 1993).

The majority of studies aimed at defining the transmembrane topography of colicins have been carried out on the 20-kDa C-terminal channel-forming domains of colicins A and E1. The experiments described below have instead focused on identifying all regions of intact colicin Ia (70 kDa), a less well-studied member of this colicin family, that associate with and are protected by a target membrane. The strategy employed in this study was to proteolytically digest all accessible regions of membrane-associated colicin Ia and then to identify the protected peptides using a combination of gel chromatography, N-terminal sequencing, and mass spectrometric analysis. Proteolysis was carried out with pepsin, both because it functions at acidic pH and because it cleaves relatively nonspecifically (Sachdev & Fruton, 1970), thereby allowing cleavage close to the membrane surface.

Proteases have been successfully used to identify transmembranous regions of a variety of proteins [reviewed in Jennings (1989)]. Recently, proteolysis of the C-terminal channel-forming domain of colicin E1 bound to liposomes has placed new constraints on the voltage-independent membrane-associated conformation of this channel (Zhang & Cramer,

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1992). Two previous studies have used mass spectrometric methods to identify and sequence small proteolytic fragments from acetylcholine receptor in membrane topography mapping experiments (Moore et al., 1989; Poulter et al., 1989). The present study takes advantage of electrospray ionization (ESI)[1] mass spectrometry [for a review, see Fenn et al. (1990)] in combination with gas-phase Edman sequencing to rapidly and precisely determine both the N- and C-termini of relatively large membrane-protected peptides generated by proteolysis of colicin Ia in the presence of membranes. We have identified two major regions of colicin Ia that are protected by negatively charged membranes at low pH, in the absence of a transmembrane voltage. Within these regions, residues lying at the N- and C-termini of protected peptides necessarily map to the cis side of the lipid bilayer, thus placing constraints on the structure of colicin Ia in this membrane-associated conformation. These results are discussed in the context of the recently solved low-resolution structure of the soluble form of colicin Ia (Ghosh, 1992).

MATERIALS AND METHODS

Liposome Preparation. Reverse-phase evaporation vesicles (REV's) were prepared by the method of Szoka and Papahadjopoulos (1978) using 1 mL of diethyl ether, 1 mL of 50 mM ammonium acetate buffer, pH 4.1, and 10 μmol of either purified (Kagawa & Racker, 1971) asolectin (Associated Concentrates, NY), egg PC (Avanti Polar Lipids, AL), or *E. coli* lipid extract (Avanti Polar Lipids).

Pepsin Digestion and Peptide Separation. Colicin Ia was expressed and purified from *E. coli* JK365 (Mel & Stroud, 1993). Typically, 7 nmol of colicin Ia was added to liposomes to a final concentration of 5.4 μM at a lipid:protein molar ratio of 1500:1, in 50 mM ammonium acetate, pH 4.1. After a 5-min incubation at room temperature, pepsin was added to a final pepsin:colicin molar ratio of 3:1, and samples were incubated at 37 °C. Within 2 min of the indicated time points, the digestion was quenched by precipitation of pepsin using organic solvents (Wessel & Flugge, 1984), a procedure which also extracts the lipids, as follows: 70–110-μL aliquots of the digestion mixture were added to 400 μL of methanol; 200 μL of chloroform and 300 μL of water were then added, and the resulting mixture was centrifuged for 1 min at 9000*g* in a microfuge. This yielded a two-phase system in which pepsin and colicin Ia membrane-protected fragments could be seen as a white precipitate at the interface. The upper phase was discarded, and the lower phase was mixed with 300 μL of methanol, vortexed, and then centrifuged for 2 min. This resulted in a pellet containing colicin Ia peptides and pepsin. The methanol mixture was discarded, and the pellet was washed with an additional 300 μL of methanol. This mixture was vortexed, and the protein was pelleted by a final 2-min centrifugation, after which the methanol was discarded. Remaining drops of methanol were allowed to evaporate in a hood. Dried pellets were then resolubilized in 10 μL of 0.25% SDS in 50 mM sodium phosphate buffer, pH 7.8, and 10 μL of Serva Blue G sample buffer (Serva Feinbiochemica, Heidelberg) for 1 h or more and then separated on 16% Tricine–SDS–PAGE gels (Schagger & von Jagow, 1987) which afford improved separation of components of M_r <14 000. Since these gels are run at pH values >8 and as pepsin is irreversibly inactivated at pH values >6 (Wilkinson, 1986), pepsin remains inactive during electrophoresis.

The experiment comparing colicin Ia digestion products in the presence versus the absence of asolectin liposomes was carried out as described above, with the exception of the organic precipitation procedure. In the presence of lipids, the lipid: protein molar ratio was 1500:1, and the molar pepsin:colicin ratio was 3:1, whereas in the absence of lipids the molar pepsin: colicin ratio was 2:1. At the indicated times, approximately 4 μg of colicin was added directly to 10 μL of 2× sample buffer and then loaded immediately onto a 16% SDS–PAGE gel (Laemmli, 1970). N-Terminal sequence and mass spectrometric analyses were not carried out on these samples.

Identification of N-Terminal Sequences and Masses of Protected Fragments. For N-terminal sequence analysis of protected fragments, gels were blotted onto Immobilon-P transfer membranes (Millipore, Bedford, MA) for 90 min at 100 mA, using a TE 70 SemiPhor semi-dry transfer unit (Hoefer Scientific Instruments, CA) and sequenced directly from Immobilon using Edman degradation. The number of residues sequenced for each gel band is indicated in Table I. For mass spectrometric analysis, protein bands were electroeluted from gels using a Model 422 Electro-Eluter (Bio-Rad Laboratories, Richmond, CA) in 50 mM ammonium bicarbonate/0.1% SDS buffer at 60 mA for 90 min. SDS was removed from dried samples using the precipitation procedure described above (Wessel & Flugge, 1984). Precipitated peptides were lyophilized and sonicated in 5 μL of formic acid and 3 μL of hexafluoroisopropyl alcohol and then immediately analyzed by ESI mass spectrometry. On the basis of an estimated protein recovery of 25% after electroelution, precipitation, and resolubilization, the maximum amount of sample available for mass spectrometric analysis was estimated to be 500 pmol (actual amounts were not determined).

Mass Spectrometry. ESI mass spectrometry was carried out on a Fisons/VG Bio-Q mass spectrometer, using a 50:50 (v/v) mixture of acetonitrile and a 2% aqueous acetic acid solution. Samples were injected in the formic acid/hexafluoroisopropyl alcohol solution described above. Resolution was set at approximately 500 (50% valley), and typically 10 8-s scans over m/z range 500–1800 were acquired and averaged.

RESULTS

Regions of Colicin Ia Are Protected from Proteolysis upon Association with Membranes. Using proteolysis as a probe of membrane-associated structure, specific regions of colicin Ia that are resistant to pepsin digestion after association with negatively charged membranes at low pH were identified. After vigorous proteolysis (pepsin:colicin Ia molar ratio of 3:1 for 1 h at 37 °C), several protein fragments remain protected in the presence of asolectin liposomes for at least 60 min (Figure 1, lanes 1–5). Three major digestion products, at approximate molecular masses of 23, 15, and 13 kDa, as well as a smear of lower molecular mass components remain protected (Figure 1, lane 5). In contrast, in the absence of liposomes, no protected regions are detectable after 10 min of digestion despite a decreased pepsin:colicin ratio, indicating that colicin Ia is rapidly proteolyzed (Figure 1, lanes 6–9). The differences between digestions in the presence and absence of lipid are not due to inhibition of pepsin activity by lipid. This was shown in a separate experiment where the extent of cleavage of a pepsin substrate (sequence ATLNFPISPW) in the presence and absence of asolectin lipids was found to be identical (data not shown). The protective effect appears to be specific to negatively charged lipids, as colicin Ia digested

[1] Abbreviations: CD, circular dichroism; Da, dalton(s); mA, milliamp(s); PA, phosphatidic acid; PC, egg phosphatidylcholine; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; PI, phosphatidylinositol; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; ESI, electrospray ionization.

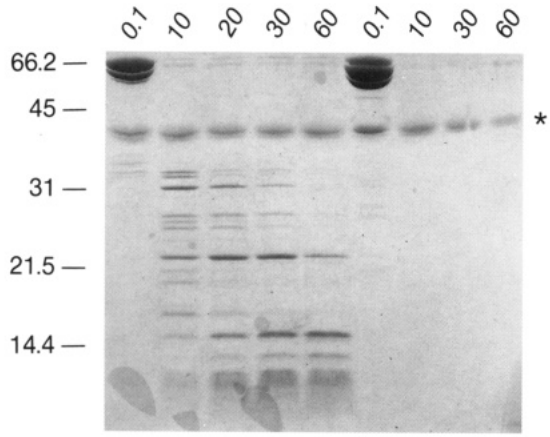

FIGURE 1: Comparison of pepsin digestion of colicin Ia in the presence versus the absence of asolectin liposomes. In the presence of lipids (lanes 1–5), the digestion was carried out at a lipid:protein molar ratio of 1500:1 and a 3:1 molar pepsin:colicin ratio, and in the absence of lipids (lanes 6–9), the pepsin:colicin molar ratio was 2:1. Approximately 4 μg of colicin was loaded onto each lane. Molecular mass standards (in kDa) are shown on the left, the pepsin band is indicated with an asterisk, and digestion times are indicated in minutes across the top. The lanes labeled 0.1 min reflect aliquots taken immediately after pepsin addition. N-Terminal sequence and mass spectrometric analyses were not carried out on these samples.

in the presence of vesicles made from neutral egg PC results in no detectable protected regions (data not shown), a result which was identical to that observed with soluble colicin Ia in the absence of membranes (Figure 1, lanes 6–9). Since colicin Ia will release the contents of negatively charged asolectin liposomes but not those of liposomes made from the neutral lipid egg PC (Mel & Stroud, 1993), the fragments that are protected after associating with negatively charged membranes are likely to be those portions of the molecule that are functionally important in membrane attachment, membrane insertion, or ion-channel formation.

Identification of Protein Sequences Protected by the Membrane. Several of the major colicin Ia fragments that remain protected by negatively charged asolectin membranes after 2 h of pepsin digestion have been identified. These fragments, labeled A–E (Figure 2A), correspond to peptide fragments of approximate molecular mass 20, 14, 10, ~9, and ~8 kDa, as determined by SDS–Tricine gel electrophoresis. [The 20- and 14-kDa bands contain the same peptide as the 23- and 15-kDa bands of Figure 1, lane 5, on the basis of N-terminal sequence analysis (data not shown).] The same pattern of protection is observed if pepsin proteolysis is carried out in liposomes made from *E. coli* rather than asolectin lipids, as fragments corresponding to A–E (Figure 2B, see arrows) are visible after 30 min of digestion (Figure 2B, lane 2). This indicates that colicin Ia associates with both types of membranes in a similar conformation. The rate of proteolysis is more rapid in the presence of *E. coli* liposomes, however; bands A and B disappear after 2 h in *E. coli* but remain protected by asolectin for up to 3 h, even with re-addition of pepsin (Figure 2C). While there were small variations in the abundance of digestion products when comparing different digestions in the presence of asolectin liposomes, the major protected fragments always included those bands labeled A–E (Figure 2A).

Bands A and B. Bands A and B (Figure 2A) are single peptides, as determined by Edman N-terminal sequence analysis and ESI mass spectrometry (Table I). The peptide in band A begins at Asp358 and has a measured mass of 18 122 ± 10 Da (Figure 3); its C-terminus is therefore Ala517 (Table I). Similarly, the single peptide in band B begins at Ser402, has a mass of 13 134 ± 10 Da, and therefore also extends to Ala517 (Table I). Both peptides contain a portion of an 18-kDa channel-forming region of colicin Ia, whose amino terminus is Asp451 and whose C-terminus is probably the C-terminus of colicin Ia (Ghosh et al., 1993). In addition, 93 and 49 residues upstream of this 18-kDa channel-forming region are protected in fragments A and B, respectively.

Band C. Band C (Figure 2A) is a mixture of several peptides (Table I), at least three of which begin at the successive positions Leu418, Leu419, and Lys420, respectively. The heterogeneity of this sample results from the relatively nonspecific proteolytic activity of pepsin, which generally cuts at either side of large aromatic or hydrophobic residues such as Phe, Met, Leu, or Trp, although there are exceptions to this specificity (Sachdev & Fruton, 1970). The presence of several different residues in each sequencing cycle (Table I) made it difficult to unequivocally identify the N-termini of components of this mixture using N-terminal sequencing alone. However, in combination with the ESI mass measurement results, the identity of the peptides in this fraction was confirmed. The mass spectrum shows several peaks, in clusters of two peaks each (Figure 4A). The masses of the smaller peaks of the first three clusters, 11 187, 11 298, and 11 412 Da (Figure 4A), correspond exactly to peptides beginning at Leu418, Leu419, and Lys420, each extending to Ala517 (Table I and Figure 4B). The first peak in the fourth cluster is 11 598 Da. While N-terminal sequence analysis was too weak to identify a fourth peptide sequence in this fraction with certainty, the 11 598-Da peptide may begin just upstream of the three peptides in this mixture, at Gln416.

The masses of the larger peaks in the first three clusters (masses 11 204, 11 313, and 11 428 Da) are all approximately 16 ± 1 Da greater than the series identified above (Figure 4A). As the sequence between Asn416 and Ala517 contains a single methionine, a residue which is readily oxidized, it is probable that these larger peaks also correspond to fraction C peptides, each containing methionine sulfoxide.

Band D. Band D (Figure 2A) is also a mixture of peptides, two of which begin at Ala79 and Tyr80, respectively (Table I). Residues consistent with a third peptide beginning at Ser85 were also present during sequencing, though in lesser amounts (Table I). Peptides in this band have an approximate mass of 11 450 ± 250 Da, but precise masses could not be assigned due to sample heterogeneity. Peptides of 11 450 ± 250 Da that begin at Ala79 and Tyr80 would extend to between Ala179 and Arg189.

Band E. Band E is very heterogeneous as determined by N-terminal sequencing, with too many peptides present to identify any with absolute certainty. However, peptides beginning at Ser85, Ala86, Gln87, and Glu521 best account for the majority of residues observed by N-terminal sequencing (Table I). As bands D and E are not well resolved by electrophoresis, several peptides were common to both bands (peptides beginning at Ala79 and Tyr80).

The mass spectrum of fraction E also reflected a large degree of heterogeneity, and specific masses could not be assigned to individual peptides. However, the peptides do fall within a mass range of 11 700 ± 400 Da. Therefore, the C-termini of the protected fragments beginning at Ser85, Ala86, and Gln87 would extend to the region between Glu184 and Gln191. A peptide beginning at Glu521 and extending to the C-terminus of colicin Ia would have a mass of 11 356 Da, consistent with the observed mass range in this fraction.

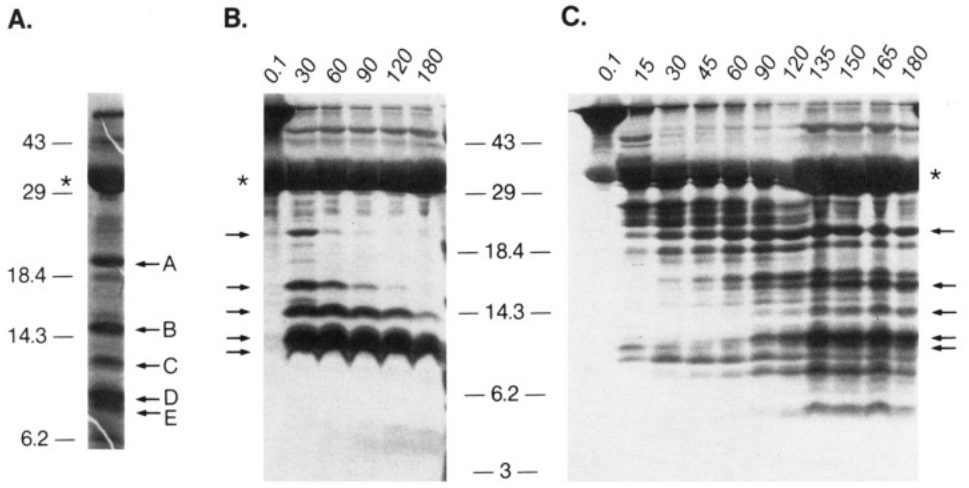

FIGURE 2: **(A) Membrane-protected fragments of colicin Ia generated after 2 h of pepsin digestion in the presence of asolectin liposomes.** The N- and C-termini of peptides in bands labeled A–E have been identified using N-terminal sequence analysis and mass spectrometry (see text). (B) Time course of colicin Ia proteolysis in liposomes made from *E. coli* lipids. (C) Time course of digestion in liposomes made from asolectin lipids. Arrows in panels B and C indicate the protected fragments that migrate at the same mass as bands A–E. Approximately 40 μg of colicin was loaded onto each lane in panel A, and approximately 27 μg of colicin was loaded onto each lane in panels B and C. All samples were precipitated as described under Materials and Methods. The numbers at the top refer to minutes of digestion, and the pepsin band is indicated by an asterisk. Molecular mass standards (kDa) are indicated at the left of panel A and between panels B and C. At 120 min in each of the time course experiments, sufficient protease was added to achieve a final concentration of 19 μM freshly added pepsin.

Table I: N-Terminal Sequencing of Colicin Ia Peptides

	sequencing cycle[a]													
gel band	1	2	3	4	5	6	7	8	9	10	pmol[b]	observed mass (Da)	calculated C-terminus	calculated mass (Da)
A	**D** 358	**V**	**E**	**G**	**D**	–	–	–	–	–	4.3 (0.3)	18122 ± 10	Ala517	18118
B	**S** 402	**A**	**R**	**T**	**N**	–	–	–	–	–	4.6 (0.1)	13134 ± 10	Ala517	13134
C	L 418	**L**	**K**	**E**	**K**	**E**	**N**	**I**	**R**	**N**	1.7 (0.6)	11412 ± 10	Ala517	11412
	L 419	**K**	**E**	**K**	**E**	**N**	**I**	**R**	**N**	**Q**	1.3 (0.1)	11298 ± 10	Ala517	11299
	K 420	**E**	**K**	**E**	**N**	**I**	**R**	**N**	**Q**	**L**	0.8 (0.1)	11187 ± 10	Ala517	11186
D	**A** 79	**Y**	**K**	**N**	**T**	**L**	**S**	–	–	–	2.3 (0.1)	11450 ± 250	Ala179–Arg189	ND[d]
	Y 80	**K**	**N**	**T**	**L**	**S**	**A**	–	–	–	3.2 (0.3)	same	same	ND
	S 85	*A*	*Q*	*Q*	( )	*E*	*N*	–	–	–	1.2 (0.4)	same	same	ND
E	*S* 85	*A*	*Q*	*Q*	( )	*E*	*N*	*R*	*N*	( )	*c*	11700 ± 400	Glu184–Gln191	ND
	A 86	*Q*	*Q*	*K*	*E*	*N*	*E*	*N*	( )	*R*	*c*	same	same	ND
	Q 87	*Q*	*K*	*E*	*N*	*E*	*N*	( )	*R*	*T*	*c*	same	same	ND
	E 521	*S*	*V*	*K*	*L*	*S*	*D*	*I*	*S*	*S*	*c*	same	Ile626	11356
	Y 80	( )	*N*	*T*	*L*	*S*	*A*	*Q*	*Q*	( )	*c*	same	Glu184–Gln191	ND
	A 79	*Y*	*K*	*N*	*T*	*L*	*S*	*A*	*Q*	*Q*	*c*	same	same	ND

[a] Uppercase boldface letters represent those sequences identified with certainty. Nonboldface italic letters identify sequences that are likely present in bands D and E, with the residues in parentheses too weak to identify. The number under the first residue of each peptide identifies the position of that residue in the colicin Ia sequence. [b] The number of picomoles of each peptide was calculated by first determining the initial yield (assuming an average repetitive yield of 0.925) for the first peptide of each series, and calculating residue carryover for the subsequent peptides in each series. [c] Each of these peptides is estimated to be present at 0.5–1 pmol. [d] Not determined. The single peptides in bands A and B comprised >95% of all material sequenced, and those in bands C, D, and E together comprised >80% of all material sequenced in each case.

Summary of Protected Regions. A summary of the protected peptides is presented in Figure 5A. Peptides identified with certainty span a region which begins at Asp358 and continues through Ala517, as well as a region which spans ~100 residues of the N-terminal domain beginning at Ala79. Sequencing data are also consistent with a protected peptide beginning at Glu521 and continuing to the C-terminus of the molecule. In contrast, two regions (residues 1–77 and residues 191–358) contain no protected peptides, indicating that these regions are not membrane-associated. The approximate locations of the translocation, receptor-binding, and channel-forming domains are indicated in Figure 5B.

DISCUSSION

We have identified two major regions of colicin Ia that are protected from proteolysis after association with negatively

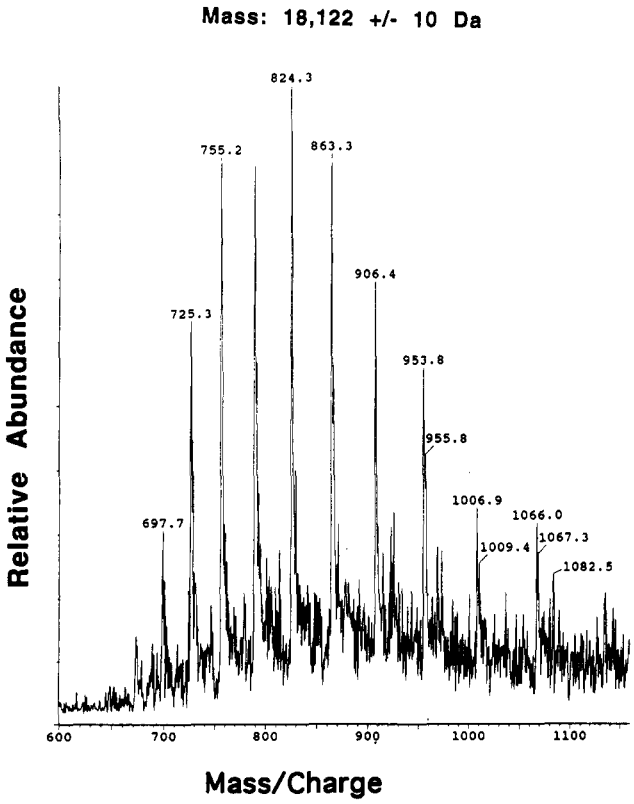

FIGURE 3: ESI mass spectrum of the single peptide in band A. The family of peaks observed arises from the same peptide with different numbers of attached protons. The measured mass of this peptide was 18 122 ± 10 Da.

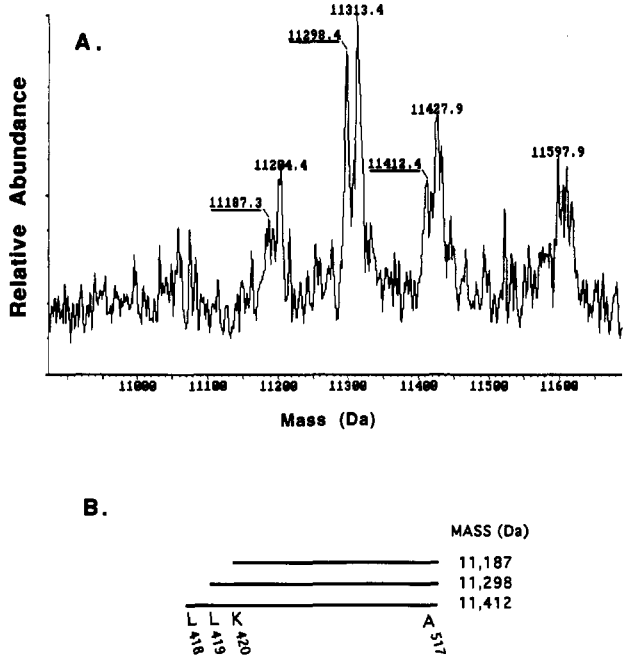

FIGURE 4: (A) Deconvoluted (Mann et al., 1989) ESI mass spectrum of peptides in band C. The three clusters represent pairs of peptides which differ by 16 ± 1 Da. The second peak in each cluster is likely an oxidized version of the first peak in each cluster. Underlined masses correspond to peptides illustrated in part B. (B) Schematic of the N- and C-termini of the protected peptides from band C.

charged asolectin membranes, the largest of which begins at Glu358 and likely continues to the C-terminus of colicin Ia. Within this region lies a C-terminal fragment (Asp451–Ile626) which forms ion-conducting channels in planar lipid bilayers (Ghosh et al., 1993). The conductance properties of these

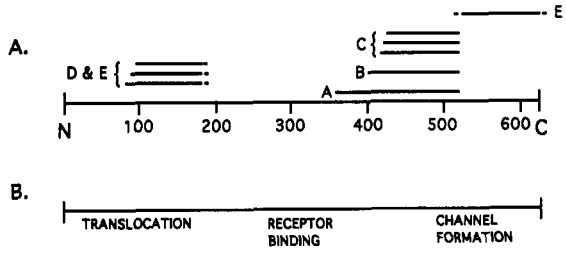

FIGURE 5: (A) Schematic of protected peptides after 2 h of pepsin digestion in the presence of asolectin liposomes. The solid lines represent termini identified with certainty, and the dashed lines represent those that were not. (B) Approximate locations of putative domains.

channels differ substantially from those of whole colicin Ia channels, however, suggesting that residues other than Asp451–Ile626 are involved in channel formation. As we have shown that the 93 residues lying directly upstream of Asp451 are tightly associated with negatively charged membranes, it seems likely that these residues play a role in channel formation. This is consistent with the recently solved X-ray structure of whole colicin Ia, which reveals α-helical protein density immediately adjacent to a 10-helix bundle (Ghosh, 1992), the motif identified as the channel-forming domains of both colicin A and colicin E1 (Parker et al., 1989, 1992; Wormald et al., 1990).

The involvement of residues other than those in the C-terminal domain in channel formation has been suggested for colicins A and E1. Part of the receptor-binding domain of colicin A is thought to interact with the channel-forming domain in a pH-dependent fashion, based on fusion and surface pressure studies in planar lipid bilayers and liposomes (Collarini et al., 1987; Frenette et al., 1989). Furthermore, an interaction between the C-terminal channel-forming domain and the central domain of colicin A has been identified using colicin A–E1 hybrid molecules (Benedetti et al., 1991). Proteolytic digestion of colicin E1 in the presence and absence of a transmembrane voltage has shown that a region upstream of the channel-forming fragment affects channel-gating (Raymond et al., 1986; Slatin et al., 1986). While regions adjacent to the C-terminal domain (in the linear sequence) have been implicated in membrane insertion in each of the above studies, these colicin Ia data provide the first evidence that a portion of the N-terminal domain is membrane-associated as well.

The precise length of the protected region in the N-terminal domain of colicin Ia is not completely defined, though residues 79–~190 are resistant to pepsin proteolysis. The fact that part of the putative N-terminal translocation domain is protected by membranes suggests that this region may be located near the channel-forming domain in the three-dimensional structure of colicin Ia. When the C-terminal domain inserts into membranes to form channels, the protected region of the N-terminal domain may be forced to lie next to the membrane because of structural constraints and thus be protected from proteolysis. In support of this idea, Ser171, which lies within this protected region, appears to be located near the end of an α-helix that is adjacent to the channel-forming domain in the X-ray crystal structure (Ghosh, 1992).

Colicin Ia associates with membranes in the absence of a transmembrane voltage in these experiments. The voltage-independent membrane-associated conformation of colicins A and E1 has been defined as a "closed"-channel state. This state is characterized by large portions of the protein being exposed to the outer or cis side of liposomes or lipid bilayers, as shown by proteolysis (Slatin et al., 1986), fluorescence

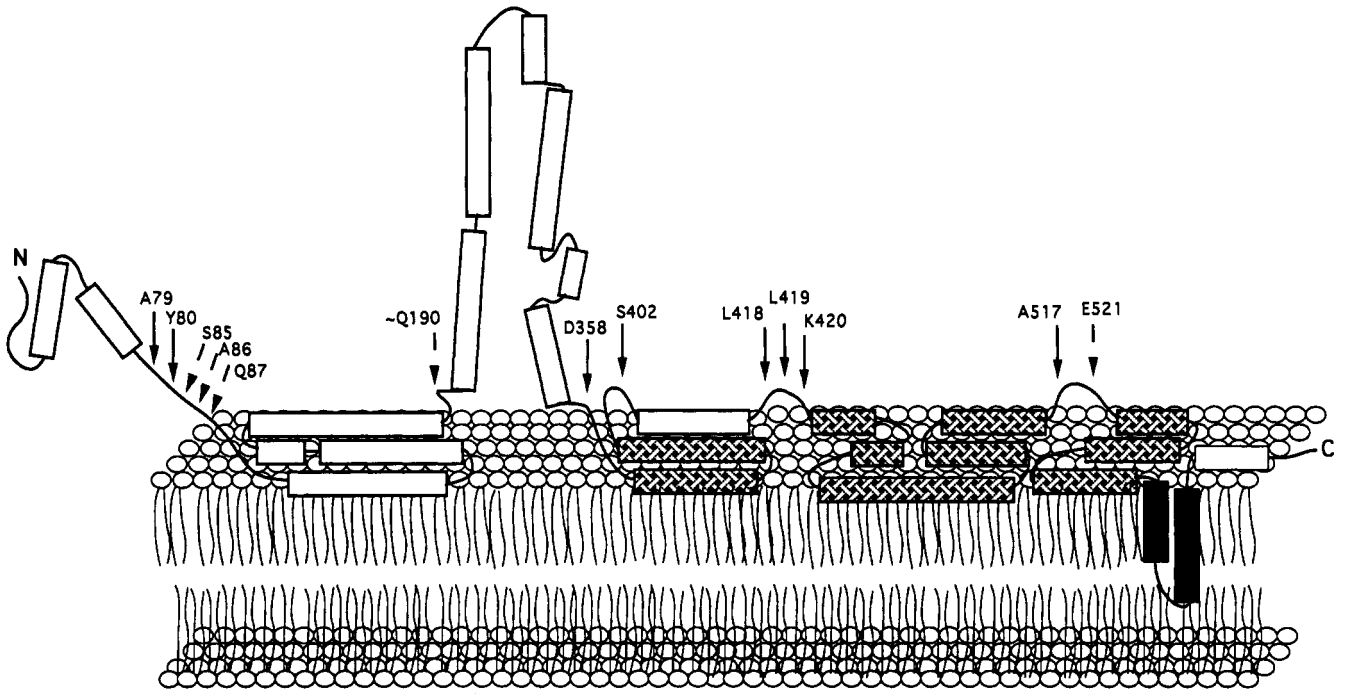

FIGURE 6: Model of colicin Ia in its membrane-associated conformation. Hydrophobic helices are black, amphipathic helices are cross-hatched, and polar helices are white. The residue numbers and solid arrows indicate the location of definite proteolytic sites while the dashed arrows are likely proteolytic sites.

spectroscopy (Lakey et al., 1991a,b), and time-resolved studies of spin-labeled mutants (Shin et al., 1993). One model suggests that colicins lie on the membrane surface in an "umbrella" conformation (Parker et al., 1990). This is in contrast to an "open"-channel state, in which portions of the protein are driven into the lipid bilayer in response to a transmembrane voltage, as measured by proteolysis (Raymond et al., 1986) or labeling with a lipophilic probe (Merrill & Cramer, 1990). Thus, we infer that colicin Ia is probably in the intermediate voltage-independent closed-channel state in our experiments, with the majority of protein associated with the surface of the lipid bilayer rather than inserted across the lipid bilayer. It is interesting to note that, on the basis of our results, the amphipathic α-helices that will eventually form the channel are already protected by the membrane in the intermediate, voltage-independent conformation, poised to insert into the membrane upon application of a transmembrane voltage.

We have generated a model for the voltage-independent membrane-associated conformation of colicin Ia (Figure 6). The overall structure is α-helical, based on circular dichroism of colicin Ia in the presence of a hydrophobic detergent environment (Mel & Stroud, 1993). The lengths of the helices are based on the X-ray crystal structure of the channel fragment of the related molecule colicin A (Parker et al., 1989, 1992) and on secondary structure prediction (Finer-Moore & Stroud, 1984), with one exception. This exception is residues 517–521 of colicin Ia, which lie within helix 4 of colicin A according to the sequence alignment of Parker et al. (1989). As pepsin definitely cuts at Ala517 and probably cuts at Glu521, helix 4 of colicin A is instead modeled as a loop region in colicin Ia. Colicin E1 residues (Leu416–Ala417) corresponding to residues 520 and 521 of colicin Ia were also cleaved in similar topography mapping experiments (Zhang & Cramer, 1992), further supporting the model that this region is a protease-sensitive loop. By analogy with colicins A and E1, the stretch of 39 hydrophobic residues, Thr573–Ile612, likely spans the membrane as an α-helical hairpin, even in the absence of transmembrane voltage (Massotte et al., 1989; Song et al., 1991). This helical hairpin is not sufficiently long to span the lipid bilayer completely, however, because colicin Ia carries a deletion of 10 residues within this region, when compared with colicin A. Finally, all proteolytic cut sites are indicated with arrows and residue numbers, and are modeled as loop regions between helices.

Three of the protected peptides, A, B, and C, share a common C-terminus (Ala517), but have different N-termini (Asp358, Ser402, Leu418, Leu419, and Lys420). Different pepsin cleavage rates at different sites probably account for the formation of subfragments of a larger protected fragment. Differences in cleavage rates were also observed when comparing proteolysis in the presence of liposomes made from *E. coli* rather than with asolectin lipids. This result can be explained by differential access of pepsin to the surface of these vesicles. The *E. coli* lipid mixture is composed of three major lipid components, PE, PG, and cardiolipin, which together constitute 96% of the membrane lipids (Gennis, 1989). In contrast, asolectin contains a larger variety of components, including ~77% phospholipids (a mixture of PA, PC, PE, and PI), 13% glycolipids, and 9% plant sugars (data supplied by Associated Concentrates). As sugar groups tend to be large and bulky, the surface of liposomes made from asolectin will likely be less accessible to pepsin than that of liposomes made from *E. coli* lipids alone. This could result in a decreased rate of proteolysis in the presence of asolectin lipids.

By coupling mass spectrometry with Edman N-terminal sequence analysis, we have identified both the N- and C-termini of colicin Ia peptides that are membrane-associated in the absence of a voltage. These data suggest that the channel-forming domain of colicin Ia extends beyond the electrophysiologically defined 18-kDa channel-forming fragment (Ghosh et al., 1993). Furthermore, for the first time, we find that a portion of the N-terminal domain of a channel-forming colicin is closely associated with membranes as well. Thus, in the case of colicin Ia, through studying the whole molecule rather than a channel-forming fragment, we have gained new

insight into the possible mechanism of the insertion of this water-soluble protein into membranes.

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