Membrane Topography of ColE1 Gene Products: the Hydrophobic Anchor of the Colicin E1 Channel Is a Helical Hairpin

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Received 26 October 1990/Accepted 19 February 1991

The paucity of crystallographic data on the structure of intrinsic membrane proteins necessitates the development of additional techniques to probe their structures. The colicin E1 ion channel domain contains one prominent hydrophobic region near its COOH terminus that has been proposed to be an anchor for the assembly of the channel. Saturation site-directed mutagenesis of the hydrophobic anchor region of the colicin E1 ion channel was used to probe whether it spanned the bilayer once or twice. A nonpolar amino acid was replaced by a charged residue in 29 mutations made at 26 positions in the channel domain. Substitution of the charged amino acid at all positions except those in the center of the hydrophobic region and the periphery of the hydrophobic region caused a large decrease in the cytotoxicity of the purified mutant colicin E1 protein. This result implies that the hydrophobic domain spans the membrane bilayer twice in a helical hairpin loop, with the center of this domain residing in an aqueous or polar phase. The lengths of the *trans*-membrane helices appear to be approximately 18 and 16 residues. The absence of significant changes in ion selectivity in five of nine mutants indicated that these mutations did not cause a large change in the channel structure. The ion selectivity changes in four mutants and those previously documented for the flanking Lys residues imply that the hydrophobic hairpin is part of the channel lumen. Water may “abhor” the hydrophobic side of the channel, explaining the small effects of residue charge changes on ion selectivity.

Colicin E1 is a bactericidal plasmid-encoded protein whose lethal action is a consequence of its ability to form a highly conductive, monovalent, voltage-gated ion channel in the *Escherichia coli* cytoplasmic membrane. The manner in which the membrane channel is assembled and gated is of interest for studies on the mechanism of action of toxins, protein import, and voltage-gated channels (8). Colicin E1 is one of six bactericidal channel-forming colicins (E1, Ia, Ib, A, B, and N), the COOH-terminal channel domains of which have an appreciable level of primary sequence identity (Fig. 1) (8, 24). The crystal structure determination of the water-soluble form of the 204-residue thermolytic channel peptide of colicin A, determined and refined to 0.25-nm resolution, revealed the presence of 10 α -helices (24). Helices 8 and 9, which are buried in the core of the soluble peptide, have been hypothesized to be inserted into the membrane bilayer as a hydrophobic α -helical hairpin, thereby providing an anchor for insertion of the other helices needed to form the final channel structure (7, 24). This hypothesis has not yet been tested by topographical experiments, although the conclusion that Asp-473 of the channel resides near the *cis* side of the membrane (15) is consistent with a helical hairpin model.

It is known that the COOH terminus of the channel is positioned on the *cis* side of the membrane (7, 38), that its secondary structure in the membrane is predominantly α -helical (25) and probably monomeric (reviewed in reference 8), and that the channel peptide segment Ala-425 to Trp-460 is inserted into the membrane bilayer in the presence of a membrane potential (19). The lengths of uncharged helices 8 and 9 in the soluble colicin A peptide are 16 and 17 residues, respectively. These peptide segments are seemingly some-

what short when compared with the membrane-spanning α -helices of the two membrane proteins, the bacterial photosynthetic reaction center and bacteriorhodopsin, which have been crystallized and solved by X-ray and EM diffraction analysis, respectively (10, 14, 28). For the soluble colicin A peptide, helices 8 and 9 and the connecting 6-residue turn segment constitute approximately 39 residues of a 49-residue hydrophobic domain. A major difference between the sequences of colicins A and E1 is that the hydrophobic domain of the latter, as well as that of colicins Ia and Ib, is shorter and consists of 35 to 39 residues. The actual value for the colicin E1 channel is dependent on whether the domain is taken as Ala-474 to Ile-508 or Ala-471 to Asp-509 (Fig. 1). Thus, it is of interest to determine the topographical arrangement of the hydrophobic domain of the colicin ion channels in the membrane, and the structure of the shorter hydrophobic domain of colicin E1 is the focus of the present study.

MATERIALS AND METHODS

Strains. *E. coli* JM101 (40) was used as a host for most of the cloning experiments and as an indicator strain for the spot test of cytotoxicity. Strain DM1187 *lexA* (35), obtained from E. Tessman and I. Tessman, was the source for the overexpression of mutant colicin E1. *E. coli* MV1190 (*dut*⁺ *ung*⁺) and CJ236 (*dut* *ung*), obtained through Bio-Rad, were used for mutagenesis (16). Helper phage M13K07 (36) was obtained from Promega (Madison, Wis.).

Construction of recombinant plasmids pM13cea and pSKE1(–). (i) **pM13cea.** The 1.3-kb *Sma*I-*Eco*RI fragment of the ColE1 plasmid that spans most, but not all, of the *cea* gene from bases 5358 to 6646 (5, 39) was cleaved by restriction digestion, separated on an agarose gel, excised, and further purified with GeneClean (Bio 101 Inc., La Jolla,

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FIG. 1. Sequences of the channel-forming domains of colicins E1, Ia, Ib, A, B, and N (numbering system for colicin E1, one-letter residue code), aligned as described by Parker et al. (24). The sources of the original sequences are summarized in reference 8. *, Positions identical in all six sequences or identical in five with a conservative substitution in the sixth. The major hydrophobic segment, Ala-471 to Ile-508, is enclosed by a bracket. This hydrophobic segment, 38 or 49 residues, defines two subgroups, E1, Ia, and Ib and (ii) A, B, and N. The α -helices defined in the solved structure of the colicin A channel peptide are marked, as are the NH_2 termini of the channel peptides obtained by tryptic (Tr), thermolytic (Th), and cyanogen bromide (CNBr) cleavage.

Calif.). M13mp11 phage DNA (20) was linearized with *EcoRI* and *SmaI* to introduce compatible sites for ligation with the 1.3-kb *cea* gene fragment. Ligation of these two fragments created a new M13 construct, pM13*cea*, screened by restriction digestion of candidate plasmids that were isolated by a modified alkaline lysis procedure (21a). Cells harboring pM13*cea* were grown overnight, single-stranded pM13*cea* DNA was isolated from the phage pellet, and the presence and orientation of the insert were confirmed by sequencing of single-stranded pM13*cea*.

(ii) pSKE1(-). The ColE1 plasmid was digested with *StuI* adjacent to bases 2570 and 4784 and by *NruI*, which cuts next to base 478 (5, 39), generating a 1.7-kb blunt-end fragment which included the *cea* gene with its promoter and the *imm* gene. The pBluescript II SK(+) phagemid (Stratagene, La Jolla, Calif.) was treated with *SmaI* to create blunt ends, the 1.7-kb fragment and the linearized vector were ligated to create pSKE1(-), the correct insertion was confirmed by restriction digestion, and pSKE1(-) was transformed into CJ236 cells. CJ236 cells harboring pSKE1(-) were grown to the mid-log phase and infected with helper phage M13K07 (36) at a multiplicity of infection of 20. Phages containing single-stranded pSKE1(-) were sedimented from the cell supernatant, and single-stranded DNA

was isolated for mutagenesis from the phage pellet. The orientation of the insert was determined by sequencing single-stranded pSKE1(-).

Site-directed mutagenesis. (i) **Taylor-Ott-Eckstein method (34).** With a mononucleotide mixture including dCTPaS instead of dCTP (Amersham kit RPN1523), the heteroduplex was treated with *NciI* to create nicks in the parental strand. Exonuclease III was used to digest the parental strand, including the mutation site, and the digested region was closed by DNA polymerase I and T4 ligase, resulting in the synthesis of the mutant homoduplex. Mutant screening was done by DNA sequencing. Once a mutant was found, the 1.3-kb *SmaI-EcoRI* fragment containing a mutation was isolated from an agarose gel and ligated with the large fragment of pDMS630 (ColE1 derivative with an ampicillin resistance marker) lacking the wild-type 1.3-kb *SmaI-EcoRI cea* gene fragment (32). Colonies that grew on ampicillin plates after ligation and transformation were restreaked, and isolated colonies were picked and grown overnight in 3 ml of 2 $\times$ YT medium containing 16 g of tryptone (Difco) per liter, 10 g of yeast extract per liter, and 5 g of NaCl per liter. Plasmid DNA was isolated, and the swap of the mutation from the pM13*cea* plasmid to the pDMS630 plasmid was confirmed by double-stranded DNA sequencing.

(ii) **Kunkel method (16).** We used two strains, MV1190 (*dut⁺ ung⁺*) and CJ236 (*dut ung*), and the pSKE1(−) plasmid (170-3576; Bio-Rad) (2a). pSKE1(−) was transformed into CJ236, and single-stranded DNA was isolated from the phage pellet by infection of the cells with helper phage M13K07 (36). The degree of uracil incorporation into single-stranded pSKE1(−) DNA isolated from CJ236 was tested by simultaneous infection of MV1190 and CJ236 with pSKE1(−)-containing M13 phage. M13 phage harboring pSKE1(−) with incorporated uracil yielded at least five times more colonies when infecting CJ236. After the mutant oligonucleotide was annealed with the template, native T7 DNA polymerase (2) was incubated (30 min, 37°C) to synthesize the mutant strand in the presence of dTTP, the closed heteroduplex was transformed into MV1190, several colonies were picked, and plasmid DNA was isolated from each. Mutants were identified by double-stranded DNA sequencing of the pSKE1(−) plasmids.

DNA sequencing. DNA sequencing for the screening of mutants was done in a model S2 apparatus (Bethesda Research Laboratories). Sequenase (USB, Cleveland, Ohio), a modified T7 DNA polymerase (33), was used in both single- and double-stranded DNA sequencing. The sequencing reaction was done in accordance with the protocol in the Sequenase kit (70770). For double-stranded DNA sequencing, double-stranded template DNA was denatured by the alkaline denaturation method (6) for annealing with the primer.

Synthetic oligonucleotide preparation. All oligonucleotides used for the mutagenesis were constructed in the Laboratory for Macromolecular Structure at Purdue University. Seventeen mutants were made by use of a separate oligonucleotide for each desired mutation. Normally, a 19-mer was used for introducing mismatches at one or two sites and a 30-mer was used for more mismatches. In addition, 12 mutants were generated from two "degenerate primers" (23); 5 mutants were obtained from one and 7 mutants were obtained from the other. The degenerate primers were made by the addition of a mixture of bases at more than one targeted site within the primer, with the base ratio calculated to optimize the generation of single mutants (17). Some of the crude oligonucleotides were purified with an oligonucleotide purification cartridge (400771; Applied Biosystems, Foster City, Calif.), and some oligonucleotides were used directly for mutagenesis without purification.

Purification of mutant colicins. Instead of using mitomycin C to induce the overproduction of colicin E1, we used the *lexA* strain DM1187 for the overexpression of the *cea* gene. After the introduction of the mutant plasmid in the form of pSKE1(−) or pDMS630 transformed into DM1187, mutant colonies were picked either from a fresh plate or from a cell stock frozen quickly with 10% glycerol at −70°C. The cells were inoculated into 250 ml of 2× YT medium directly, grown overnight, sedimented, and resuspended in lysis buffer (50 mM sodium phosphate buffer with 0.1 M NaCl–1 mM octylglucoside [pH 6.8] and a 1 mM concentration of each of the following protease inhibitors: α -toluenesulfonyl fluoride, ϵ -amino-*n*-caproic acid, iodoacetamide, and EDTA). The resuspended cells were passed through a French press, and the lysates were sedimented at 10,000 × *g* (15 min). Clear lysates were dialyzed against 50 mM sodium phosphate buffer (pH 6.8) for at least 3 h to equilibrate them with the chromatography buffer, passed through 0.20- μ m-pore-size syringe filters, and loaded in small aliquots on a Mono-S cation-exchange column attached to a fast protein liquid chromatography unit (Pharmacia, Uppsala, Sweden).

The salt gradient (0 to 1 M NaCl) was programmed so that the column flow rate was 1 ml/min, 1 M NaCl was achieved in 30 min, and the mutant colicin was eluted at 0.3 M NaCl. Each mutant peak was collected in one or more fractions, and each fraction was run on a sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) Phast gel system (Pharmacia) to identify the most pure fraction. Selected mutant fractions were pooled and filtered, and the optical density at 280 nm was measured to determine the concentration with a molar extinction coefficient of 0.7 (3). Cell lysates and mutant fractions collected from the column were stored at 0°C. The purity of each mutant was again tested after filtration on the SDS-PAGE Phast gel system. Cytotoxicity was measured either immediately after purification or with samples that had been frozen at −70°C.

Cytotoxicity. The cell strain used as the colicin E1-sensitive indicator, JM101, was grown overnight to the stationary phase, and 100 μ l was spread on a 2× YT plate. From a 10- μ g/ml stock solution, fivefold serial dilutions to 16 ng/ml were assayed. The range of 10 μ g/ml to 16 ng/ml was chosen because it was found that all mutants showed observable activities within this range. Twenty microliters from each dilution was spotted directly on a plate that had been overlaid with the indicator cells. The plates were incubated at 37°C overnight, and the lowest dilution of mutant colicin that yielded complete clearing at the spotted site was used to calculate the specific activity of the mutant being tested.

Planar bilayer measurements of channel ion selectivity. Colicin was added to one side (*cis*) of a planar membrane formed by apposing monolayers (21). The membrane separated the 1 M and 100 mM salt solutions (see Table 1). For experiments at pH 6 and pH 4, protein was added to the low- and high-salt sides of the membrane, respectively. As the channels are cation selective at pH 6 and anion selective at pH 4, this protocol for the addition of protein ensured that the channels were open at the measured reversal potential. The channels were opened by applying negative voltages, where the sign of a voltage refers to that of the *trans* compartment; the *cis* compartment was taken as 0 mV. We routinely checked that the direction of the current switched when the applied voltage was changed from one sign of the reversal potential to the other.

RESULTS

Saturation mutagenesis of the COOH-terminal hydrophobic segment of the colicin E1 channel domain was used to determine the number of membrane-spanning α -helices in this segment. The rationale was that replacement of a hydrophobic by a charged amino acid in a membrane-spanning α -helix that has a generally hydrophobic nature should have a tendency to thermodynamically destabilize that helix in the lipid bilayer, impeding the insertion of the hydrophobic anchor region into the membrane and reducing its cytotoxicity. The magnitude of this destabilization may depend on the length of the *trans*-membrane helices, so that relatively long helices (e.g., bacteriorhodopsin [1]) with larger hydrophobic interactions may be able to accommodate some charged residues in the hydrophobic *trans*-membrane helices. On the other hand, insertion of a charged amino acid into the 10- to 15-residue hydrophobic segment of bacterial signal peptides is almost always deleterious (13). Thus, if the *trans*-membrane α -helices in the colicin E1 channel are relatively short, as seems likely from the small size (~150 to 190 residues [4, 7]) of the channel-forming

FIG. 2. Models for possible membrane folding of the 35- to 38-residue hydrophobic domain of the colicin E1 channel peptide. (A) Single membrane-spanning helix. Only a fraction (~ 20 residues) of the hydrophobic domain spans the bilayer. The amino acids in the region of residue 490 are positioned near the center of the nonpolar core of the bilayer for a ~ 20 -residue helix centered in the 38-residue hydrophobic domain. The single membrane-spanning helix could also extend, for example, from approximately residues 470 to 475 to residues 490 to 495 or from residues 485 to 490 to residues 505 to 509, in which case residues on one side would be in the nonpolar environment and those on the other would be in the polar environment or aqueous phase. (B) α -Helical hairpin model. Two membrane-spanning helices are shown with only the residues in the turn region around residue 490 in the polar environment. (C) Model of a channel assumed to be monomeric (8). Shown are a hydrophobic helical hairpin anchor (black) and an amphipathic segment (stippled) that is translocated into the membrane in the presence of a membrane potential, $\Delta\psi$ (19). Also shown is a helical hairpin segment (white), upstream (NH₂) of the voltage sensor, that is thought from labeling experiments (19) to be inserted into the bilayer in the absence of a potential.

peptides, the *trans*-membrane channel helices may not be able to accommodate a mutation to a charged residue.

The closer the charged residue is to the hydrophobic center of the bilayer, the region of lowest polarity, the greater should be the destabilizing effect. Thus, if the hydrophobic domain, extending from approximately residue Ala-471 to Asp-509, were distributed symmetrically across the membrane and spanned the bilayer once, then the central region around residues 489 and 490 would be in the most hydrophobic central region of the membrane (Fig. 2A). Replacement of nonpolar residues with charged amino acids near positions 489 and 490 in the center of the hydrophobic domain would then cause the largest destabilization and loss of cytotoxic activity. If the membrane-spanning helix crossed the bilayer once but was asymmetrically positioned on one side or the other side of the 38-residue hydrophobic domain (e.g., either the segment from 471 to 489 or that from 490 to 508 spanned the membrane bilayer), then the substi-

tution of charges at positions in the spanning helix, but not in the other helix, would cause destabilization and loss of cytotoxic activity. However, if the hydrophobic segment spanned the membrane twice as an α -helical hairpin, the residues near the center of the hydrophobic region (e.g., residues 489 to 493, Gly-Thr-Thr-Leu-Gly) would form a hairpin turn situated near a polar interface (Fig. 2B). From considerations of electrostatic interactions with the membrane dielectric, replacement of one of the residues in this turn by a charged amino acid would have a smaller effect on cytotoxic activity.

The relative cytotoxicities of 29 colicin E1 mutants arising from the substitution of a charged amino acid at the site of each of 26 different nonpolar residues in the hydrophobic domain is shown in Fig. 3. In each case, the colicin protein ($>90\%$ pure) was isolated and the cytotoxicity was assayed relative to that of the wild type. Substitution of a charged for a nonpolar residue at most of the positions in the hydropho-

FIG. 3. Cytotoxicity caused by the single charge mutations in the COOH-terminal hydrophobic region of colicin E1. The two uppercase letters following the residue numbers indicate the wild-type residue (first letter) and the mutation introduced (second letter), using the one-letter amino acid code (charged residues: D, aspartate; E, glutamate; K, lysine; R, arginine). The size of each bar indicates the specific activity of the mutant on the basis of at least three independent cytotoxicity tests. The relative activity of each mutant, as compared with a wild-type activity of 100%, was determined from the ratio of the lowest wild-type protein concentration to the lowest mutant protein concentration that would cause clearing of the bacterial lawn on a petri dish in a spot test. For mutants with activities of less than 1% of the wild-type activity, a minimum value of 1% is shown on the graph so that the bars will be visible. Following are the exact values of activities of the mutants relative to that of the wild type (100%), with standard deviations (number of trials): 471A→K, 1.6% ± 1.6% (4); 472A→K, 14% ± 7.8% (2); 474A→E, 29% ± 16% (11); 477S→R, 11% ± 7% (4); 478Y→K, 1% ± 0.7% (7); 479V→D, 0% ± 0% (4); 480V→D, 0% ± 0% (4); 481A→D, 3% ± 1.1% (4); 482L→K, 0.4% ± 0.3% (3); 483L→R, 3% ± 1.4% (3); 485S→R, 9% ± 8% (4); 486L→K, 6% ± 3% (3); 487L→R, 0.4% ± 0.3% (4); 489G→E, 66% ± 31% (10); 489G→K, 30% ± 14% (5); 490T→K, 28% ± 14% (3); 491T→K, 30% ± 15% (11); 492L→K, 2% ± 1.6% (3); 493G→D, 74% ± 24% (10); 494I→K, 1% ± 0.6% (3); 496G→D, 6% ± 3.6% (6); 496G→R, 0.1% ± 0.3% (6); 497I→K, 0.25% ± 0.1% (3); 499I→K, 0% ± 0% (3); 501T→E, 1.0% ± 0.7% (3); 502G→E, 1.0% ± 0.7% (3); 502G→K, 5% ± 6% (2); 504L→K, 0.7% ± 1.0% (3); and 507Y→K, 54% ± 22% (6). Both an acidic residue and a basic residue were substituted at residues 489, 496, and 502. The *in vitro* channel activity of the COOH-terminal channel peptide derived from 15 and 6 mutants was also measured on liposomes and planar bilayers, respectively. It was not possible to reach a conclusion about the pattern of *in vitro* activities of the hydrophobic domain mutants because the ranges of activities of the peptides derived from the mutants were smaller and the variances were larger, relative to those of the wild type, than obtained for cytotoxicities.

bic domain resulted in low activity (see the legend to Fig. 3 for actual values). The one region that had substantially higher activity was the central segment consisting of residues 489 to 493. Five of the six mutants with mutations made in this region (two mutations, Lys and Glu, were made at position 489) had activities much greater than did those with mutations made at positions upstream (NH₂) to residue 474 and downstream (COOH) to residue 507. It is clear that residues 489 to 493 are unique in the 38-residue hydrophobic domain, and we conclude that they are close to a polar milieu. The mutants with charges at residues 474 and 507 also had higher activities, in agreement with the helical hairpin model of Fig. 2B that places these sites close to the polar interface region. All of the cytotoxicity data would be accounted for if the hydrophobic domain of the colicin E1 channel peptide spans the membrane bilayer twice in a helical hairpin structure.

The ion selectivity of the channel was measured at different pH values to determine (i) whether the mutant channel peptides can form a correct structure and (ii) whether the changes in charge alter the ion selectivity. Colicin E1 has a

characteristic pH-dependent selectivity, varying from cation selective at pH 6 to anion selective at pH 4 (26). The ion selectivity at pH 4 of five of the mutants was not significantly different from that of the wild type (Table 1), implying that the structure of the channel formed by the mutants was similar to that formed by the wild type. Therefore, the lower activity of the mutants was interpreted as being due to a decreased probability of forming the correct structure. Four mutations, 487L→R and 489G→E, along with 501T→E and 502G→E, measured previously (29), caused decreased anion selectivity at pH 4 (Table 1) (see legend to Fig. 3 for an explanation of designations).

DISCUSSION

The helical hairpin structure for the COOH-terminal hydrophobic domain agrees with the analogous α -helical hairpin structure proposed for the colicin A channel peptide made of helices 8 and 9 (16 and 17 residues, respectively; Fig. 1) on the basis of crystallographic analysis (24). The concept that this hydrophobic domain may serve as an

TABLE 1. Reversal potentials and ion selectivities of the wild type and mutants with a single charge substitution in the hydrophobic domain

Protein	Reversal potential ^a (no. of membranes) at pH:		Ion selectivity ^b at pH:	
	6.0 ^c	4.0 ^d	6.0 (P _{K⁺} /P _{Cl⁻})	4.0 (P _{Cl⁻} /P _{Na⁺})
Wild type	36.0 ± 2.0 (3)	-29.1 ± 2.4 (9)	6.7	4.4
D-481		-27.0 ± 2.0 (3)		3.9
R-485		-27.0 ± 1.0 (3)		3.9
R-487		-23.3 ± 0.6 (3)		3.2
E-489		-23.4 ± 0.6 (5)		3.2
K-489	37.5 ± 0.7 (2)		7.5	
D-493	34.5 ± 0.7 (2)	-27.7 ± 3.0 (7)	6.1	4.0
D-496	37.5 ± 3.5 (2)	-26.1 ± 1.4 (7)	7.5	3.7
E-501		-21.8 ± 3.6 (4) ^e		2.9
E-502		-20.0 ± 1.4 (2) ^e		2.7

^a Mean ± standard deviation.^b P, Membrane permeability of an ion species.^c Measured in 1 M KCl versus 0.1 M KCl (with 3 mM CaCl₂-5 mM dimethylglutaric acid-0.1 mM EDTA) bathing asolectin planar membranes. Channels are selective for monovalent cations over anions.^d Measured in 1 M NaCl versus 0.1 M NaCl (with 3 mM CaCl₂-10 mM dimethylglutaric acid-0.1 mM EDTA) bathing asolectin planar membranes. Channels were selective for Cl⁻ over Na⁺, as indicated by negative values.^e From Shirabe et al. (29).

anchor for insertion of the colicin channel into the membrane has been discussed previously for colicin A (22) and for colicin E1 (7, 13, 27, 29-31). The tightness of the turn made by residues 489 to 493 does not seem to be a structural problem, as ~5 residues can function as extramembrane turn regions connecting *trans*-membrane helices in bacteriorhodopsin (1, 14).

The anchor region and the 35-residue segment (Ala-425 to Trp-460) that is inserted into the membrane bilayer in a voltage-dependent manner (19) can then form two α -helical hairpins of the channel domain (Fig. 2C). The labeling experiments that defined the voltage-dependent insertion of this segment also indicated that another segment of the channel further upstream was inserted into the membrane (19). Therefore, the channel is drawn in Fig. 2C as a three-hairpin structure containing six helices. The arguments for a four- versus a six-helix structure were discussed previously (16). (No data are available for the helix-helix interactions suggested in the original discussion of α -helical hairpins in membranes [12] that are implicit in an ion channel made of α -helices.)

One question concerning the interpretation of the data is whether the results can be understood in terms of structural changes in the soluble protein. An expected effect of charge substitution in the hydrophobic protein core is protein unfolding. Such an unfolding was documented for the substitution of the Glu residue at position 501 or 502 (29). However, partial unfolding of the colicin channel peptide itself was associated with an increase in activity (18). Therefore, the inhibitory effects of the mutations in the hydrophobic segment were associated with the membrane-bound state of the colicin.

The Glu-Lys-Lys and Asp-Lys-Lys sequences at positions 468 to 470 and 509, 510, and 512, respectively, indicate the approximate locations of the ends of the hydrophobic hairpin region. It is also possible that the hydrophobic domain is even shorter and ends at Asp-473 on its NH₂-terminal side. However, we note the following. (i) The rules for insertion and translocation of carboxylate residues into and across

membranes are not as strict as are those for positively charged residues (37), perhaps because it has been shown that carboxylates can be protonated in membranes and thereby neutralized, with an effective pK_a of 9 to 10 (23). Perhaps for this reason, there is a net excess of bacteriorhodopsin carboxylates in the bilayer of the membrane of *Halobacterium halobium* (14). (ii) The problem of accommodating a charged residue near the bilayer periphery is less difficult than doing so in the center because of the higher dielectric constant near the membrane surface and the possibility of packing a charged side chain toward the aqueous phase (10). Therefore, we propose that the α -carbons of Asp-473 and Asp-509 are located in the bilayer. The resulting uncharged hairpin helices would then appear to be no longer than 18 (471 to 488) and 16 (494 to 509) residues.

It is still possible that the helices in the α -helical hairpin anchor model are too short to span the membrane bilayer. These proposed *trans*-membrane helices, particularly that nearer the COOH terminus, appear to be substantially shorter than the membrane-spanning α -helices of the photosynthetic reaction center (10, 28) and bacteriorhodopsin (14) and the "rule of thumb" of approximately >20 residues reported in the literature (e.g., 11) for the length of the uncharged domains of such helices. We wish to make two comments on this rule. (i) It is not true for bacteriorhodopsin, in which four of the seven helices have charge-free membrane-spanning segments of ≤ 18 residues (14), or for the photosynthetic reaction center, in which the charge-free region of the membrane-spanning subunits can be as short as 2.5 nm (10, 28). These charge-interrupted structures for bacteriorhodopsin and the photosynthetic reaction center may arise from their charge translocation function. However, shorter uninterrupted hydrophobic α -helical membrane-spanning segments have been noted for other proteins. For example, 16 nonpolar residues are sufficient to anchor the gene III protein of the coliphage $\phi 1$ protein in the cytoplasmic membrane (9). (ii) One should be careful about calculating the length of the *trans*-membrane helices on the basis of the number of α -carbons in the putative spanning segment. There is a spatial separation between the amino acid α -carbon and the charged group of its side chain (0.7 nm for Arg). This separation may allow the side chains of charged residues near the termini of the helices to pack toward the aqueous interface of the membrane (10). The lengths of the membrane-spanning helices are still shorter than expected from the documented structural data. Thus, we conclude that the lengths of the hydrophobic *trans*-membrane helices implicated by these studies are shorter than the 20 residues expected from the literature.

The changes in ion selectivity caused by the mutations (Table 1) do not clearly establish that the α -helices that are proposed to form an anchor for the channel also form part of the channel lumen in contact with water and the ion flow. Only four of the nine mutations caused a change in ion selectivity at pH 4, and for one of these (487L→R), the decreased anion selectivity was in a direction opposite to that expected from electrostatic considerations. On the other hand, Lys-510 and Lys-512 on the COOH end of the hydrophobic segment affect ion selectivity (30). Additional studies on ion selectivity in the hydrophobic segment as well as the upstream amphipathic regions are needed. Presently, it appears that the hydrophobic helical hairpin is part of the channel lumen but that the water may tend to "abhor" the hydrophobic side of the channel. In addition, charge changes may alter the ion selectivity through structural perturbation as well as electrostatic effects.

In summary, the picture emerging is that the channel-forming domain of colicin E1 has a hybrid structure (Fig. 2C): the membrane-spanning helices are a mixture of hydrophobic (helices 8 and 9; colicin A notation [24]) and amphipathic (e.g., a helical hairpin spanning residues 420 to 460 [19]) helices. If two hydrophobic helices formed part of the lining of a channel structure containing four to six helices, then the lumen of the channel would be less favorable for ion permeation than if all of the channel helices were amphipathic. This hypothesis would explain the relatively low single-channel conductance (≤ 20 pS in 1 M salt) (4, 7) associated with a channel lumen of appreciable size (≥ 0.8 nm) (26).

ACKNOWLEDGMENTS

We are grateful to S. Schendel and D. Ok for skilled technical assistance and to J. Hollister for precise editorial work on the manuscript.

This work was supported by grants from the National Institutes of Health (GM-18457 to W.A.C. and GM-27367 to F.S.C.).

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