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A Very Short Peptide Makes a Voltage-Dependent Ion Channel: The Critical Length of the Channel Domain of Colicin E1

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ABSTRACT **Cleavage of colicin E1 molecules with a variety of proteases or with cyanogen bromide (CNBr) generates COOH-terminal fragments which have channel-forming activity similar to that of intact colicin in planar lipid bilayer membranes. The smallest channel-forming fragment obtained by CNBr cleavage of the wild-type molecule consists of the C-terminal 152 amino acids. By the use of oligonucleotide-directed mutagenesis, we have made nine mutants along this 152 amino acid peptide, in which an amino acid was replaced by methionine in order to create a new CNBr cleavage site. The smallest of the CNBr-cleaved C-terminal fragments with channel-forming activity, in planar bilayer membranes, was generated by cleavage at new Met position 428 and has 94 amino acids, whereas a 75 amino acid peptide produced by cleavage of a new Met at position 447 did not have channel activity. The NH_2-terminus of the channel-forming domain of colicin E1 appears therefore to lie between residues 428 and 447. Since, however, the last six C-terminal residues of the colicin can be removed without changing activity, the number of amino acids necessary to form the channel is 88 or less. In addition, the unique Cys residue in colicin E1 was replaced by Gly, and nine mutants were then made with Cys placed at sequential locations along the peptide for eventual use as sulfhydryl attachment sites to determine the local environment of the replaced amino acid. In the course of making 21 mutants, eight charged residues have been replaced by uncharged Met or Cys without changing the biological activity of the intact molecule.**

It has been proposed previously that the conformation of the colicin E1 channel is a barrel formed from five or six α-helices, each having 20 amino acids spanning the membrane and two to four residues making the turn at the boundary of the membrane. Our finding that 88 amino acids can make an active channel, combined with recently reported stoichiometric evidence that the channel is a monomer excludes this model and adds significant constraints which can be used in building a molecular model of the channel.

Key words: colicin E1, site-directed mutagenesis, ion channel.

INTRODUCTION

Colicin E1 (ColE1), a bacterial toxin, is a single polypeptide of 522 amino acids[1] which kills sensitive *Escherichia coli* cells by forming an ion channel in their inner plasma membranes. The channel causes the dissipation of the potassium gradient[2] and the discharge of the transmembrane potential,[3] and thus arrests oxidative phosphorylation.[4] When attacking a sensitive cell, the colicin protein first attaches to the vitamin B12 receptor on the outer membrane[5] and, after translocation through the outer membrane, the C-terminal domain of the molecule forms the channel in the inner plasma membrane.[6] The intact colicin molecule, as well as the C-terminal fragments obtained by various proteolytic enzymes or by cyanogen bromide (CNBr) cleavage, have been shown to produce voltage-switchable ion channels in planar lipid bilayer membranes[7,8] and in phospholipid vesicles.[9] A *cis*-positive potential is required to produce a rapid turn-on of the ionic conductance, which can then be turned off by a *cis*-negative potential; that is, the channels are voltage-switchable. Raymond et al[10] estimated the size of the colicin channel from reversal potential measurement obtained with gradients of large cations and ions across colicin-treated planar bilayers. These studies indicated that the minimum channel diameter is about 9 Å, since the channel proved permeable to ions such as NAD^- and tetraethylammonium.

The colicin protein is produced by cells carrying the ColE1 plasmid, which contains genes to encode and regulate its synthesis as well as genes to control and regulate the replication of the plasmid itself. The plasmid also encodes the production of a protein which breaks the cell open, permitting the release of the colicin,[11] and in addition it also encodes the production of an immunity protein which makes the producing cell resistant to the action of the external toxin.[12] The DNA sequence of the portion of the plasmid which

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codes for the colicin protein was reported by Yamada et al,[1] and the amino acid sequence shown in Figure 1 was deduced from it. Inselburg and Applebaum[13] isolated a number of ColE1 plasmids containing the TN3 insertion element, which includes the structural gene for β-lactamase, making cells carrying the plasmid resistant to ampicillin, and one of these was used in the present experiments.

We have previously reported that a COOH-terminal fragment produced by CNBr cleavage of the native colicin and having only 152 amino acids can form channels in the planar lipid bilayer system.[7] This result, and the activity of the shorter peptides reported here, led us and others to look critically at the possibility that the channel is formed by more than one such peptide fragment.The kinetics of cell killing, first reported by Jacob et al. in 1953,[14]showed that colicin acts as a "one-hit" agent, suggesting that only one "particle" is involved in killing sensitive cells. Similar kinetic studies have subsequently been reported both for cell killing and the induction of ionic leakage from cells and phospholipid vesicles[2,9]; Schwartz and Helinski have also shown that the molecule in solution is a monomer.[15] However, in spite of the linear kinetics, many colicin molecules were required to produce the measured effects in these experiments. For example, Bruggeman and Kayalar[16] reported a very clean linear response using ion leakage from large, 3,000-Å phospholipid vesicles; however, 10 to 1,000 molecules of colicin were required per vesicle to cover the range over which the observed effect was linear with colicin concentration. For bacterial killing, at least ten molecules are necessary to produce one killing "hit"[17] and several authors have suggested that less than 10% of the colicin molecules are active with respect to their ability to form channels.[16] However, recent experiments by Levinthal et al.[18] that used an electron spin label to measure the trans-membrane potential with a method developed by Cafiso and Hubbell[19] have demonstrated not only that all molecules are active, but also that each one can discharge the potential across the phospholipid membranes of small sonicated vesicles with diameters of about 400 Å. Thus, direct stoichiometric analysis has now shown that the trans-membrane potential of *one* small vesicle is discharged by the attachment of *one* colicin molecule.

As part of our effort to determine the three-dimensional structure of the molecule as it forms the channel, we have initiated a program of site-directed mutagenesis coupled with various methods of analysis of the altered proteins and extensive molecular model building with a high speed computing system[20] and interactive computer graphics. The mutagenesis has, to date, been limited primarily to the replacement of various amino acid codons by the codon *atg* for methionine or *tcg* for cysteine. The methionines are introduced to provide new CNBr cleavage sites, and the cysteines to generate new attachment sites for sulfhydryl reagents such as nitroxide groups for electron paramagnetic resonance (EPR) analysis, or molecules of different charge or size in order to study their effect on ionic conductance.

In this paper, we report the results obtained with the methionine mutants, which have permitted us to generate shorter and shorter peptides in order to test their ability to form ion channels in planar lipid bilayer membranes. In addition, we report on the biological activity of some of the cysteine mutants which shows that several charged amino acids can be changed to Cys without destroying the channel function of the molecule. Since our overall objective is to use the colicin peptide as a model system for electrostatic and conductance studies of an ion channel, we were pleasantly surprised to find that peptides obtained by cleavage after insertion of methionines at positions 428 and 516 of the wild-type protein are still able to form an active channel *in vitro*. The fact that a peptide that is certainly less than 94, and probably less than 88 amino acids, can function to form the channel rules out several otherwise plausible models for its structure and suggests experiments which can help to define the details of the channel structure.

MATERIALS AND METHODS

DNA polymerase I large fragment (Klenow fragment) was obtained from Bethesda Research Laboratories (BRL) and all other enzymes were from Biolabs; radioactive nucleotides were from DuPont (NEN) with a specific activity of 6,000 Ci/mmol for γ-P^{32}-ATP and 800 Ci/mmol for α-P^{32}-ATP. Sep-pak C18 cartridge columns were from Walters Associates; Sephadex G-50, CM-Sephadex and other column materials from Pharmacia; nitrocellulose filters from Schleicher and Schull; protein and nucleic acids molecular markers from BRL; octylglucoside from Calbiochem.; and asolectin (lecithin type II) from Sigma Chemical.

Strains

JC411 his^- met^- leu^- arg^-

514 wild type, colicin-sensitive strain

GM119 dam-3, dcm-6, metB1, thi-1, lacZ4, galK2, gtalT22, mtl-2, tonA2, tsx-1, nal^r, supE44, / F′ KM

JM105 Δ(lac-pro), thi, strA, hsdR4, / F′ traD36, proAB, $lacI^qZ$, M15

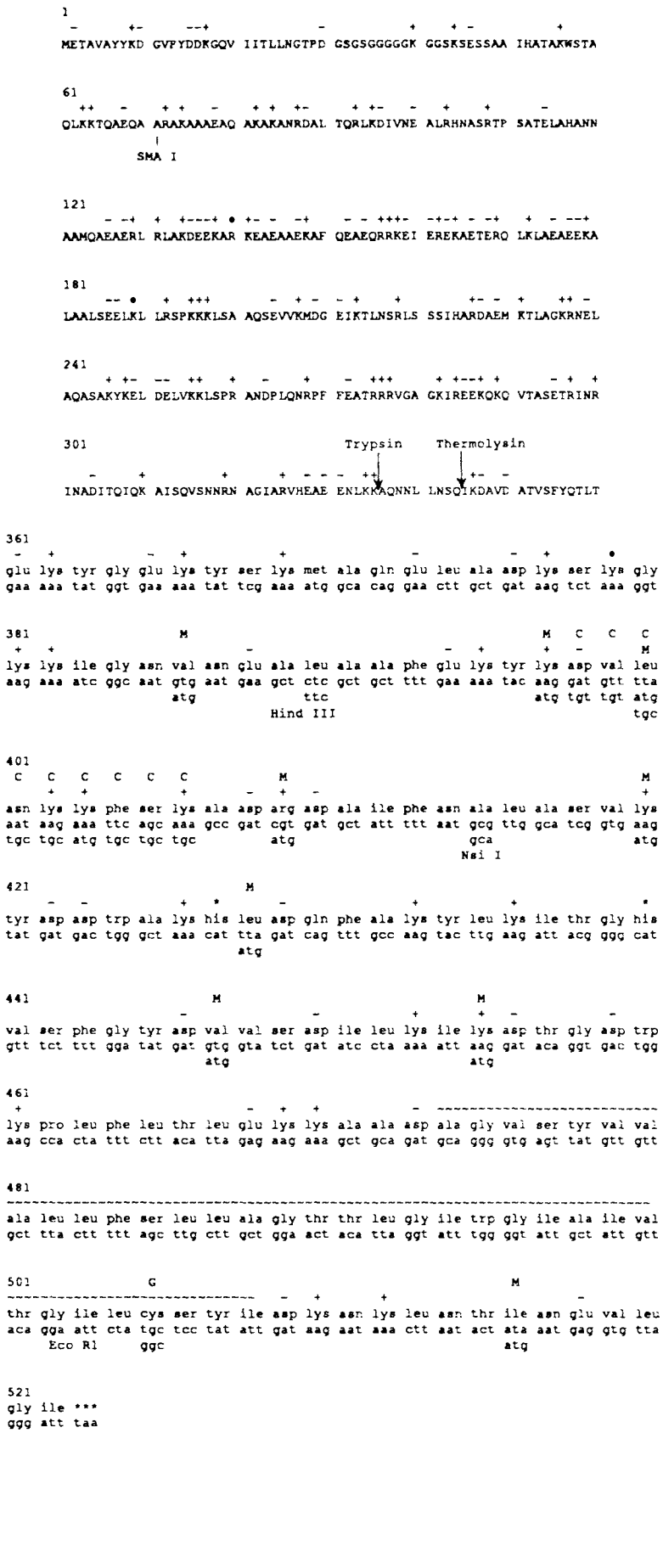
7118 Δ(lac-pro), /F' proAB, $lacI^qZ^-$, *SupE*

ColE1::Tn3 plasmid 3-1[13] was used as colicin E1 producing strains in this work, since selection for ampicillin resistance is easier and more efficient than selection for colicin immunity.

Oligonucleotide Synthesis

Oligonucleotides were synthesized using a DNA Synthesizer (Applied Biosystem Inc., Model 380A), leaving the terminal trityl group on. Following the synthesis, protecting groups were removed by incubation with fresh anhydrous ammonium hydroxide at 60°C for 6 hr. The sample was then lyophilized, purified on Sep-pak C18 cartridge columns, and resus-

COLICIN E1 AND ITS MUTANTS



pended in 80% acetic acid for 45 min at room temperature.[21] Five hundred nanograns (approximately 100 pmol) of the desired oligonucleotide were phosphorylated with T4 polynucleotide kinase and 1 μl of γ-P^{32}-ATP (equivalent to 10 μCi) before being used in further experiments.

Oligonucleotide-Directed Site-Specific Mutagenesis

Three methods were used to produce the oligonucleotide-directed mutants. The first two consisted of creating a single-strand gap in an otherwise double-stranded DNA molecule and closing the gap using the DNA polymerizing activity of the Klenow fragment with a chemicallly synthesized oligonucleotide as a primer. The synthesized fragments used for mutagenesis were 17–20 nucleotides long and were complementary to the single-stranded-wild-type DNA except for one, two, or three base differences needed to produce the desired mutation. This material is referred to as the mismatch-oligo. In the third method, oligonucleotides of either 12 or 18 nucleotides were inserted into the plasmid at the EcoR 1 site. The details of the three methods are as follows:

1. *The 3-1 plasmid DNA* was cleaved with SmaI to create blunt ends. It was then digested with Exonuclease III in order to remove nucleotides at the 3′ ends of the DNA molecules. In the conditions used [50 units ExoIII with 10 μg of 3-1 linear plasmid DNA at 32°C for 10 min], approximately 1,500–2,000 nucleotides were removed from the 3′ ends of the DNA. Then 0.8 pmol of the DNA, mixed with 50 pmol of the mismatch-oligo was denatured by heat and allowed to reanneal slowly: 10 min at 70°C, 1 hr at room temperature, 2 hr on ice. The 5′ protuding ends of the DNA were then filled by using the DNA polymerizing activity of the Klenow fragment and ligated. DNA was extracted, ethanol precipitated, and dissolved in 50 μl TE buffer (10 mM Tris, 1mM EDTA, pH 7.5). Ten microliters were used to transform competent *E. coli* JC411 cells. Unless otherwise stated, all procedures were those described in Maniatis et al. [22]

2. *Mutagenesis in M13* vectors was done following the procedure described by Bauer et al.[23] This technique uses M13mp10, which contains amber mutations within two genes essential for phage growth. A portion of the DNA encoding the ColE1 protein coming from the plasmid 3-1 was inserted into M13, after the plasmid and the phage were cut with SmaI and EcoR 1. The M13mp10-ColE1 phage was then grown in *E. Coli* GM119, a suppressor strain in which M13mp10 can grow but has a dam$^-$ mutation, preventing DNA methylation. The single-stranded DNA, carrying the wild-type ColE1 insert which was not methylated, was purified from the phage. Double-stranded M13mp10 replicative form DNA with no insert was cut with SmaI and EcoR 1, producing linear, methylated M13 DNA. A gapped heteroduplex template was constructed by mixing single-stranded unmethylated DNA with double-stranded methylated DNA having no insertion and no amber mutations. The mismatch-oligo phosphorylated at the 5′ end was added and used as primer, and the gap was closed by synthesis catalyzed by Klenow fragments and sealed with T4 DNA ligase. The heteroduplex DNA obtained is composed of one strand which is dam−, ColE1 wild type and one strand which is methylated and ColE1 mutated.

This DNA was used to transform JM 105 cells (sup−), and most of the plaques obtained contained the altered codon since the strand with the wild-type insert could not reproduce in these cells. The mutants were checked by plaque hybridization, with the radioactive mismatch-oligo as a probe, and the sequence of the DNA was determined.[24] The mutated insert was then transferred to the ColE1 plasmid by cutting with EcoR 1-SmaI and subsequent ligation. Colonies were again checked by hybridization with P^{32}-labelled probe.

3. *Some mutants were prepared by insertion of an oligonucleotide at the EcoR 1 site of colE1.* The oligonucleotides, used in these insertions, were constructed with the following constraints: the number of nucleotides inserted was a multiple of 3 in order to leave the reading frame unperturbed; part of the sequence was self-annealing and the ends were able to hybridize with the "sticky" ends left by the EcoR 1 cut. Most of the oligonucleotides inserted were 12 mers of the general form A ATT 123 4 4′3′ 2′1′, with the primed positions being complementary to the unprimed so that self-annealing occurred in solution. For example, the 12-mer sequence (5′ A ATT TGC TAG CA 3′) forms a double-stranded region from nucleotide 5 through 12 by self-annealing and has nucleotides 1 through 4 complementary to the cohesive EcoR 1 ends. The insertion of multiple fragments was prevented since the oligonucleotides had 5′-hydroxyl ends. Selection of mutants was based on the fact that plasmids with an insertion no longer had an EcoR 1 recognition site.

Mutants Selection by Colony-Hybridization

The oligonucleotide used for mutagenesis was phosphorylated with γ-P^{32}-ATP and used as a probe for hybridization. The transformed colonies, grown on ampicillin plates, were transferred to nitrocellulose paper and tested for hybridization. Positive colonies

Fig. 1. Colicin E1 and its mutants. The amino acid sequence of the wild-type colicin E1 molecule with the corresponding DNA coding sequence for amino acids 361 to 522. The numbers on the left indicate the positions of the amino acids along the polypeptide chain. From position 1 to 360, amino acids are represented by the one-letter code. Residues which are charged at pH 7.0 are marked with a plus or minus. The hydrophobic region is indicated by tildes. Arrows indicate the positions where thermolysin and trypsin cleave the protein and result in C-terminal fragments resistant to further proteolysis. Where mutations have been introduced, the mutated codon is inscribed below and the substituted amino acid above in a capital letter using the one letter code. Two new restriction endonuclease sites have been created—Hind III and Nsi I, both unique in the entire ColE1 plasmid containing the transposon TN3 (plasmid 3-1). These two sites will be used later for introducing new mutations in the channel region.

were selected from replica plates, the plasmid DNA extracted from those colonies was used to transform host cells a second time, and another selection was performed following the same procedure. Positive colonies from the second selection were grown for colicin production.

Colicin Protein Production

L broth with ampicillin was inoculated with an overnight culture of colicin-producing cells, and the cultures were grown to exponential phase (about 10^8–10^9 cells/ml). Mitomycin C (0.2 mg/ml) was then added and the culture kept in the dark for another 4 hr. Cells were harvested by centrifugation, the pellet resuspended in 50 mM KH_2PO_4, PH 7.0, and the bacteria disrupted by passage through a French Press at 6,000 psi. Cellular debris were removed by centrifugation.

For large-scale preparation of wild-type or mutant colicin, the supernatant after the cell disruption was purified on a CM-Sephadex column following the procedure of Cleveland et al. [7]

Spot Test

In the spot tests used for approximate determination of colicin activity, the supernatant, before the Sephadex column purification, was diluted by factors of 10 in L broth. L plates were overlaid with 3 ml soft agar containing 0.2 ml fresh overnight culture of *514* sensitive cells, and 1 μl of the supernatant dilutions was spotted on the soft agar. After incubation at 37°C for 5 hr, clear spots could be seen where the concentration of colicin was high enough to kill the sensitive cells. Killing units were defined as the highest dilution giving a clear spot on the plate. For better comparison, dilution in twofold increments was later made after finding the approximate range of effective dilution, which was generallly between 10^5 to 10^6.

Preparation of CNBr Fragments

Before cutting with CNBr, the purified colicin was digested by thermolysin, in order to facilitate further purification of the fragment. Ten milligrams of colicin were digested with 0.1 mg of thermolysin at 37°C for 2 hr in a Tris-HCl buffer (pH 7.4) containing 5 mM $CaCl_2$. The thermolysin-resistant C–terminal fragment was purified by passage through a Sephadex G-50 column (1.5 × 90), in a buffer containing 25 mM Tris-HCl (pH 7.4) and 1 mM β-mercaptoethanol. The first protein peak eluted from the column contained the thermolysin-resistant fragment. Fractions from this peak were dialyzed in the cold against H_20 and lyophilized. This fragment after resuspension in 70% formic acid (0.5–2 mg protein/ml) was treated with CNBr at a final concentration of 20 mg/ml and then lyophilized according to Cleveland et al.[7]

SDS-Gel Electrophoresis and C-Terminal Fragments Purification Through Electroelution

To obtain good separation of small peptides, a urea-SDS gel of 15% polyacryamide[25] was used at a thickness of 3 mm. A protein sample of 200–400 μl (approximately 1 mg protein) was applied to the gel, and the electrophoresis was run at a constant voltage of about 5 V/cm, until the indicator dye (Bromophenol blue, 0.03%) ran off the gel. On the two edges of the gel, 0.5 cm were cut off and stained in a Coomassie Blue solution for a few hours, followed by destaining overnight, while the rest of the gel was wrapped and stored at 4°C. The stained edges were soaked in water to restore their original size, put back to their original places, and the strip of gel which matched the stained band was cut off with a razor and slid into a cellulosse dialysis tube (SPECTRAPOR, m.w. cutoff: 1,000). The protein was electroeluted in a horizontal gel box, at about 5 V/cm for 2 hr. The protein solution was then dialyzed against H_20. Precipitation often occurred at this stage, because of the hydrophobicity of the denatured C-terminal fragments. The solution was well mixed with a Pasteur pipette and aliquoted into Eppendorf tubes before lyophilization.

Determination of Protein Concentration

Protein concentrations were determined spectrophotometrically at 280 nm. One OD unit corresponds to 1.3 mg. of pure colicin E1 and to 0.7 mg for its C-terminal thermolysin-resistant fragment. These values were determined from the amino acid compositions and verified by Lowry's assay [26] performed for each protein sample prior to assays in planar lipid bilayer membranes.

Conductance in Lipid Bilayers

The conductance caused by the C-terminal CNBr fragment of all the mutants was assayed in phospholipid bilayers as described in our previous work.[7] Planar phospholipid bilayer membranes were formed by the union of two monolayers[27] of asolectin from which neutral lipids had been removed.[28] Monolayers were spread from 1% lipid solution in hexane, and, after evaporation of the hexane, membranes were made across a 100-μm hole in a Teflon partition, previously painted with squalene. The C-terminal fragment of colicin E1, dissolved in a 0.5% octyglucoside, was added directly to the aqueous phase on one side, after the membrane was formed. All bilayer experiments were done in symmetric solutions of 1 M KCl, 5mM $CaCl_2$, 5 mM dimethylglutaric acid, pH 3.5

RESULTS

The production of mutants by oligonucleotide-directed mutagenesis is now rapid and straightforward, especially with the use of M13 and the basic method described by Bauer et al.[23] Although there is an obvious advantage in making the mutations in the ColE1 plasmid itself, since it avoids the problem of moving the mutated fragment from M13 back to ColE1, the convenience and efficiency of the M13 system for constructing and verifying the desired mutants is so much greater that it did not seem warranted to continue using other procedures.

In general, mutants are designated by the residue they replace in the colicin molecule, the position of that amino acid in the protein, and the amino acid which replaces them. For example, the mutant Val386Met indicates that the valine codon, *gtg*, at amino acid position 386, has been changed to a methionine codon, *atg*, by a single guanine to adenine substitution. Of the 21 single codon mutations produced to date, nine of them introduced a Met codon and ten introduced or removed a Cys codon. In addition, two mutations were made to introduce new unique restriction sites (Hind III and Nsi I) in order to make the mutagenesis simpler and permit the use of a shorter single-stranded gap in the M13 insert (see Fig. 1)

Mutant Proteins

In this paper, we will discuss mainly the functional results obtained with the Met mutants introduced as new cleavage sites for CNBr. However, it is worth noting that none of the single codon mutations in this series, including four which changed a Lys to an uncharged Cys or Met, altered the biological activity of the full-length colicin protein, nor did the Met substitution change the *in vitro* activity of the 185 amino acid thermolysin fragment on lipid bilayer membranes. Furthermore, these single codon changes did not alter the protease resistance of the C-terminal domain nor the colicin yield of the *E. coli* cells induced with mitomycin. There is one mutant, however, His440Met, which has not as yet been adequately tested and which seems to cause the protein to be rapidly degraded, and it may be for this reason that we have, as yet, been unable to purify the protein. In addition, the insertion of a 12-mer which adds four new codons at the EcoR 1 site dramatically reduces the protein yield from induced cells. It seems likely that this reduction is due to incomplete folding of the C-terminal domain and subsequent proteolytic destruction of the colicin within the producing cells. This conclusion is supported by the observation that fragments shorter than intact colicin are seen on gel electrophoresis carried out for the final analysis of the protein after it has been purified on a CM-Sephadex column. Furthermore, there was approximately a threefold increase in protein yield when the mutated plasmid was used to transfect BNN103[29] cells which are defective in one of the *E. coli* proteases (lon−). As a control, the wild-type plasmid was also grown in BNN103 and produced the same yield of protein as in wild-type *E. coli* JC411[30] (gels not shown here).

In order to produce a shortened peptide and have available the alterations translated by different suppressor strains, an amber codon was created by addition of a 12-mer (5′ A ATT TGC TAG CA3′) at the EcoR 1 site. The results were ambiguous, because there was a low level of read-through of the amber codon even though various bacterial strains were used in attempts to eliminate the production of full-length colicin. In order to ensure complete translation arrest, three stop codons were inserted by the addition of 18 nucleotides (5′A ATT TTA TAA GCT TAT TA 3′) at the EcoR 1 site. In this mutant, there was no read-through, and sufficient truncated protein was produced to test biologically. It was found that it had no channel-forming or biological activity,[30] thus confirming results reported earlier[31] with *in vitro* synthesis of the same truncated peptide.

Conductance in Planar Bilayers

The ability of colicin E1 and the various peptides formed from it to induce voltage-dependent conductance in planar lipid bilayers was compared under conditions which were identical for all of the fragments. It was found that thermolysin C-terminal fragments made from each of the methionine mutants caused a voltage-dependent conductance similar to that caused by the same fragment prepared from wild-type colicin. Conductance increased at *cis*-positive voltages and leveled off at a value of approximately 5×10^4 pS per ng of protein added per ml of buffer on the *cis* side of the membrane. Negative voltage on the *cis* side turned off the conductance.

C-terminal fragments formed by cyanogen bromide treatment of the thermolysin fragments from both the wild-type and from methionine mutants were tested to determine their channel-forming activity. The methionine mutants tested were at positions 386, 397, 400, 409, 420, 428, 447, 455, 516, and the double mutants 409-516 and 420-516. All of these, except the Lys455Met and the Val447Met CNBr fragment, induced voltage-dependent conductance in bilayers. Representative conductance records are shown in Figure 2. Panel A compares current tracings from two membranes treated, respectively, with 50 ng of wild-type CNBr fragment and Tyr420Met CNBr fragment. Comparable rates of current increase were observed with the two fragments with *cis*-positive voltage and for current decrease with *cis*-negative voltage. Panel B shows single-channel records of the wild-type CNBr fragment and the Leu428Met CNBr fragment. The short records shown here are not meant to illustrate all of the open states that can be observed. A proper comparison would entail characterization of all these states, which we have not attempted. However, it is clear that the channels are at least similar, and may turn out to be essentially identical.

The results of these experiments are summarized in Figure 3. The macroscopic conductance observed with the CNBr fragments was less than that caused by the thermolysin fragments, and this may be explained by the fact that they had been through a rather harsh denaturation treatment that the thermolysin fragments were spared. Alternatively, it may be because the CNBr fragments are more insoluble than the longer, and presumably more tightly folded, thermolysin-resistant peptide.

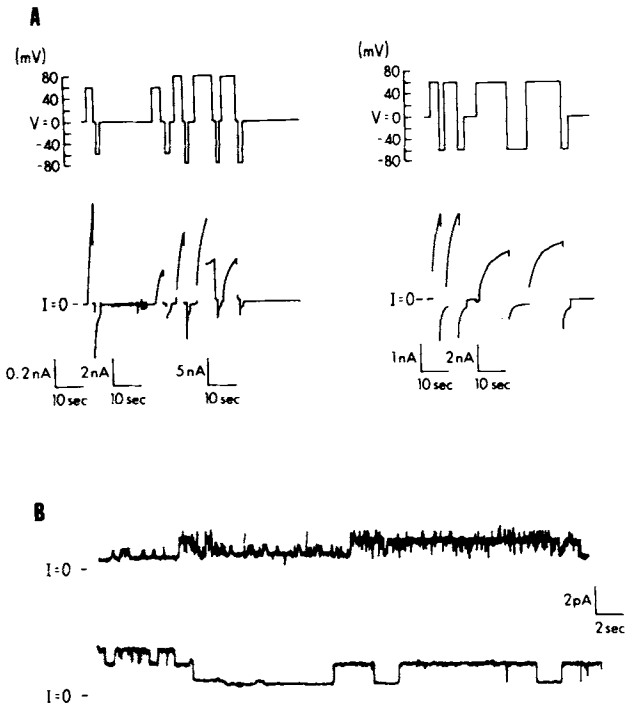


Fig. 2. Conductance records of the CNBr C-terminal fragments of wild-type and Met mutants. A: Macroscopic currents from the 152 amino acid wild-type CNBr fragment (left) and the 102 amino acid Tyr420Met CNBr fragment (right). Both asolectin membranes separated identical solutions of 1 M KCL, 5mM $CaCl_2$, 5mM dimethylglutaric acid (DMG), pH 3.5. In both experiments, 50ng of protein in 0.5% octylglucoside was added shortly before the segment of record shown was recorded. B: Individual channels of wild-type CNBr fragment (lower) and Leu428Met CNBr fragment (upper). Both membranes were made from DPPC (diphytanoylphosphatidylcholine) and separated identical solutions of 1 M KCl, 5mM $CaCl_2$, 5 mM DMG, pH 3.5. Fifty nanograms of protein in 0.5% octyl glucoside was added to each membrane several minutes before these traces were recorded. (Colicin has about 1,000-fold lower activity in neutral DPPC than in negatively charged asolectin.) The voltage is +60 mV in both records. The large current jumps in the lower trace (filtered at 30 Hz) are about 23 pS; those in upper tracer (filtered at 100 Hz) are about 20 pS.

Purity of the CNBr Fragments

The CNBr fragments tested on the planar lipid membranes were prepared by CNBr treatment of thermolysin fragments as follows. The intact colicin proteins were first cut by thermolysin and the protease-resistant C-terminal peptide of about 185 amino acids was purified on a G-50 Sephadex column. This material was then suspended in 70% formic acid and treated with CNBr. When the product was run on urea-SDS gel electrophoresis, additional fragments were frequently observed which did not correspond to those expected if all of the known methionine cleavage sites had been completely cut (Fig.4). These partial digest fragments were seen at low intensity on the gels stained with Coomassie blue, and the desired fragments, which can be seen as major bands in Figure 4, were eluted from the gels. The final material used for the electrophysiological assays was further analyzed by gel electrophoresis as shown in Figure 5.

Additional tests were carried out with two critical fragments: the shortest active one, cut at 428, and the longest inactive one, cut at 447. Neither of these two mutants contained Ile516Met, so their expected C-terminal fragment lengths were 94 and 75 amino acids, respectively. Complete cleavage at the methionines would also produce a fragment of 25 amino acids, extending from the thermolysin cut to the wild-

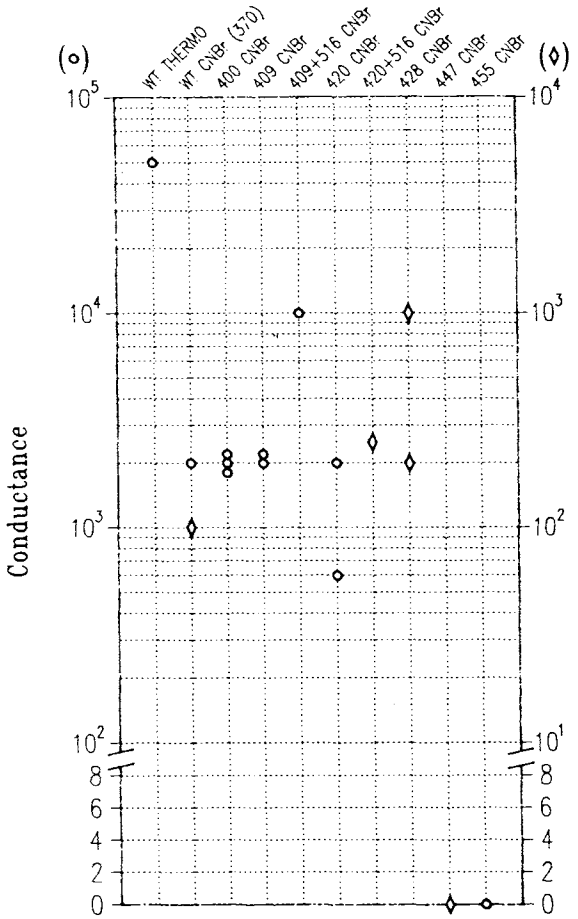


Fig. 3. The channel-forming activity of colicin E1 fragments. Data are shown for fragments from two different batches (◊, on the right scale; o, left scale). Membranes were formed from asolectin; the solution on each side was 1 M KCl, 5mM $CaCl_2$, 5 mM dimethylglutaric acid, pH = 3.5. Fragments dissolved in 0.5% octylglucoside were added, and the conductance measured was the value reached by the system at a reasonably steady state; it is expressed in pSi/ng protein. The lower part of the scale is linear and the upper part logarithmic. The numbers in parentheses represent the lengths of the peptides. Note that the two smallest CNBr fragments (from Val447Met and Lys455Met) were inactive. Membranes treated with Lys455Met or Val447Met CNBr fragment had, in some experiments, a non zero conductance (data not shown) at a level at least 100-fold lower than that of the longer fragments. Some conductance was also occasionally seen in membranes treated with material eluted from bands of the gel that represent non C-terminal protein. The extremely low level of activity in all these cases, and the fact that exactly zero activity was often seen with Val447Met and Lys455Met CNBr fragment, suggest that these fragments are themselves totally inactive, and that any residual conductance is the result of contamination by the highly active fragments. This small contamination could have occurred either during purification, subsequent handling, or from residual activity in the chamber.

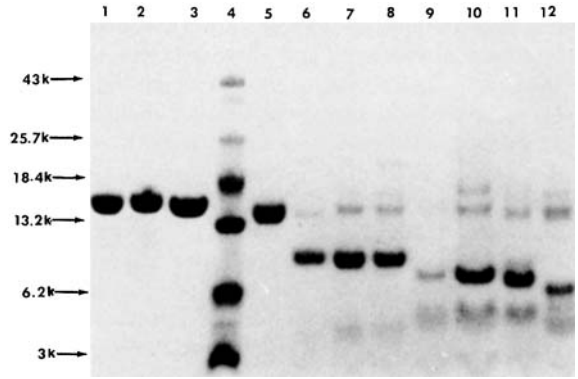


Fig. 4. Gel electrophoresis of CNBr fragments from the thermolysin-resistant C-terminal peptides before purification. The C-terminal fragments of different mutants were run on urea-SDS gel electrophoresis. Lanes 1,2, and 3 are the thermolysin-resistant fragments from mutants Arg509MET, double mutant ARG509Met, and Ile516Met, and Lys420Met, respectively. Lane 4 shows molecular weight markers with their sizes indicated on the left hand scale. Lanes 5–12 are the CNBr fragments prepared from the thermolysin-resistant piece obtained from wild-type 3-1, Lys397Met, Leu400Met1, Leu400Met2, Arg409Met1, Arg409Met2, double mutant Arg409Met and Ile516Met, and mutant Lys420Met; the numbers 1 and 2 after Met correspond to different clones of the same mutant.

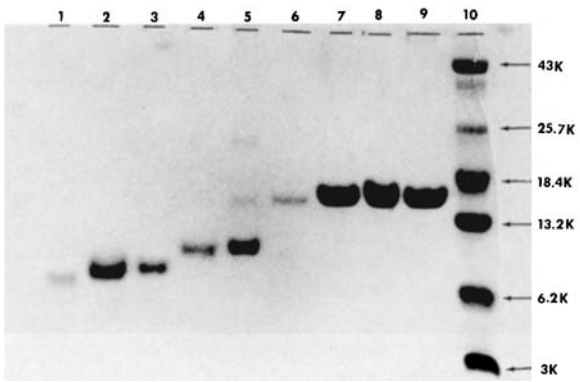


Fig. 5. Gel electrophoresis of CNBr C-terminal fragments after purification. All gels were run as described in Materials and Methods. The desired band was cut from preparative gels and rerun on a gel under the conditions indicated for Figure 4. Lanes 1–6 are CNBr fragments from mutants Lys420Met, double mutant Arg409Met and Ile516Met, Arg409Met, Lys397Met, and wild-type 3-1, respectively. The fragment in Lane 5 has not been purified. Lanes 7–9 represent the migration of the thermolysin-resistant fragment, before CNBr treatment, of Leu400Met, Val386Met, and wild-type 3-1. Lane 10 shows the molecular weight markers.

type Met at 370, and fragments of 58 and 77 amino acid extending from 370Met to 428Met and 447Met, respectively. One can also expect an active 152 amino acid fragment as the result of partial cleavage, in addition to other partial cleavage fragments. An important concern was that the observed activity of the 94 amino acid fragment might be due to contamination with larger active pieces. For the 94 and the 75 amino acid peptides, portions of the electrophoresis gel which were both ahead and behind the major band were cut out and carried through the standard elution procedure (Fig. 6). The mixture of the two short fragments from 447Met, that is, the shorter 75

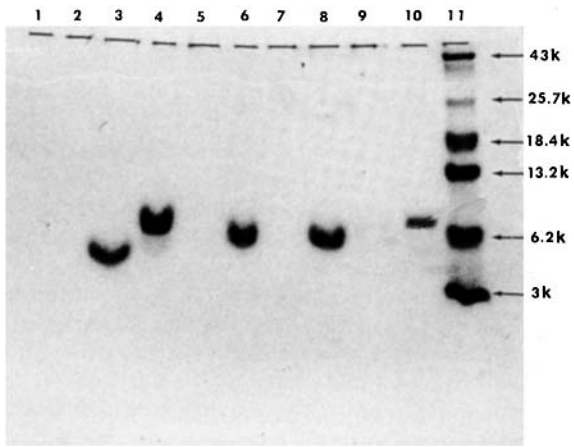


Fig. 6. Gel electrophoresis of CNBr fragments after purification of the shortest active mutant and of the longest inactive mutants. Gel showing material taken from the electrophoretic purification of the CNBr fragments from Val447Met, Leu428Met, and double mutant Lys420Met and Ile516Met. Lanes 1 and 2 were from regions of the Val447Met purification gel which ran ahead of the main band. Although *no protein was seen in these regions*, the gel slices were eluted and the eluates tested on planar membranes: no activity was observed; lane 3 was the main band of the same gel, presumably containing the 75 and the 77 amino acid fragments—the one C-terminal (75aa) and the other (77aa) from wild-type 370Met to Val447Met. This material was totally inactive in planar membranes. Lane 4 was deliberately overloaded and came from partially digested material in which the cleavage did not occur at Met370 but only at Met447, generating a 102 amino acid fragment. Lane 5 represents material which ran ahead of the main 94 amino acid band of Leu428Met, and Lane 6 shows the material from this main band. The material run on lane 5 was inactive, whereas that on lane 6 was active. This C-terminal 94 amino acid fragment was the shortest active one tested. Lanes 7–9 are CNBr fragments from the double mutant, at Lys420Met and the Ile516Met; Lane 7 and lane 9 were taken from the purification gel ahead and behind the main band, which is shown in lane 8. Only the material run in lane 8 was active. Lane 10 was a repeat of the C-terminal fragment of Arg409Met, and Lane 11 shows the molecular weight markers.

amino acid fragment mixed with the 77 long piece, and all of the material taken from the gel regions without major bands gave no evidence of channel conductance when added to planar lipid membranes. Thus, even though the active CNBr fragments produce less conductance than the thermolysin-resistant peptides, there was no confusion between the activity of the 428Met fragment and the lack of activity produced by the 447Met fragment.

Ohno-Iwashita and Imahori[6] reported that the thermolysin-resistant fragment which they found to be active in producing channels had lost ten amino acids from the C-terminal end in addition to the cleavage at position 337 in the protein sequence. The thermolysin fragment obtained and purified by us was sequenced from both its N-terminal and C-terminal ends by Dr. S. Burken and his colleaques in the Protein Facility of the College of Physicians and Surgeons at Columbia University. The thermolysin resistant fragment, produced under our conditions, retained the C-terminal amino acids of the wild-type and showed two minor N-terminal ends at positions 340 and 344 in addition to the major one at position 348. The differ-

ence between these results and those of Ohno-Iwashita and Imahori may be due to the use of different enzyme preparations or unknown differences in the digestion conditions, but, in any case, we used the Ile516Met mutant to demonstrate that at least the six amino acids at the C-terminus are not needed for channel formation. If, however, we take into account the results of Ohno-Iwashita and Imahori, then the maximum length of the channel-forming peptide would be 84 instead of 88 amino acids.

In summary, all of the C-terminal fragments formed after CNBr cleavage showed activity when tested in the planar bilayer system, with the exception of the fragments produced from the Val447Met and Lys455Met mutants. The fragments which showed activity formed channels in membranes that behaved similarly under the conditions used, although we did not search closely for subtle differences (but see Raymond et al.[32] for a comparison of whole E1, thermolysin C-terminal fragment, wild-type CNBr C-terminal fragment, and CNBr Val386Met C-terminal fragment under slightly different conditions). The failure of the 75 amino acid CNBr Val447Met and of the 67 amino acid CNBr Lys455Met fragments to form a channel is presumably due either to their missing part of the channel structure, or to an inability to carry out the transition from the attached state with no open channel to the state in which the peptide passes through the membrane to make an open channel.

DISCUSSION

Colicin Mutagenesis and Colicin Release From Cells

In this paper, we reported on the production of several site-directed mutations of the colicin E1 gene and its products. All of the mutations which alter a single amino acid codon, except one, produce proteins which are fully active when tested for their bacterial killing activity. This is true even for four mutations which change a charged amino acid to an uncharged Cys or Met and one of these mutants, Lys455Met, is in the region of the protein which we have now shown to be in the channel-forming domain. However, all of the insertions which add four codons at the EcoR 1 site, like the one mutation which changes His440 to 440 Met lead to reduced protein yield.

As indicated in the results, this reduced yield of protein from bacteria carrying certain alterations in the structural gene for colicin seems likely to result from degradation of the protein within the producing cells, because of incomplete or improper folding. In our experiments, the cells are disrupted in a French press so that all the colicin is liberated, whether it is in the cytoplasm, the periplasmic space, or loosely attached to the membrane. On the other hand, Yamada and Nakazawa[34] reported the mutagenesis of the colicin gene by chemical treatment of a 208 nucleotide single-stranded gap in otherwise double-stranded DNA. They studied eight mutated ColE1 plasmids which produced altered proteins, and of these, five produced colicin protein with three to ten amino acid alterations, and three had single amino acid changes. The major conclusion which the authors drew from their analyses was that all their mutants produced proteins which function normally, except for three which could not be exported through the inner cell membrane at the usual rate. Their conclusion is based on the assumption that the hydrophobic sequence acts as a signal sequence, and is responsible for protein release from the cell, and that cell lysis due to the kil gene is not the dominant mode of colicin release. We believe that Yamada and Nakazawa are observing the same phenomenon as we are, although our interpretations of the data are very different. Further experiments may be needed to determine which of these mechanisms, if either, is correct.

The Channel Forming Domain

An essential prerequisite to serious model building of the colicin E1 channel is the determination, as accurately as possible, of the location of the two ends of the channel-forming domain of the protein. We had previously found, from experiments with the wild-type protein, that this domain resides in 152 amino acids at the C-terminal end. In this paper, we have further delimited this region by using site-directed mutagenesis and CNBr cleavage to generate known fragments of various sizes from the protein, and by assaying the ability of these peptides to form channels in planar lipid membranes that resemble channels formed by the native protein. The mutants created in order to determine the maximum length in the channel-forming region were of two classes—those which permit a cleavage at the amino end of the presumed channel region and those which provide cleavage at the carboxy-terminal end of the colicin. Nine methionine mutants were made to produce cyanogen bromide cleavage sites beyond the 370 Met of the wild type, which had been used as the cleavage site to produce the 152 amino acids peptide.

Our major results are summarized in Figure 7. The upstream, or amino terminus, of the critical region is between residues 428 and 447, because Leu428Met CNBr can form a colicin-like channel, whereas Val447Met CNBr cannot. Likewise, the carboxy terminus of the critical region lies between residues 503 and 516, since cleavage at a methionine introduced at position 516 does not change the activity of the otherwise wild-type peptide (nor does it change the activity of other Met mutants with 2 new CNBr cleavage sites), whereas stop codons introduced at position 503 lead to an inactive protein. Thus, we can conclude that a polypeptide with only 88 amino acids (extending from Asp 429 to Asn 517) is able to form a voltage-switchable channel in phospholipid membranes. The slightly shorter peptide of 76 amino acids, bounded by residues 447 and 522, cannot form a channel in our system, presumably because it is missing crucial structural elements of the channel, or because it is

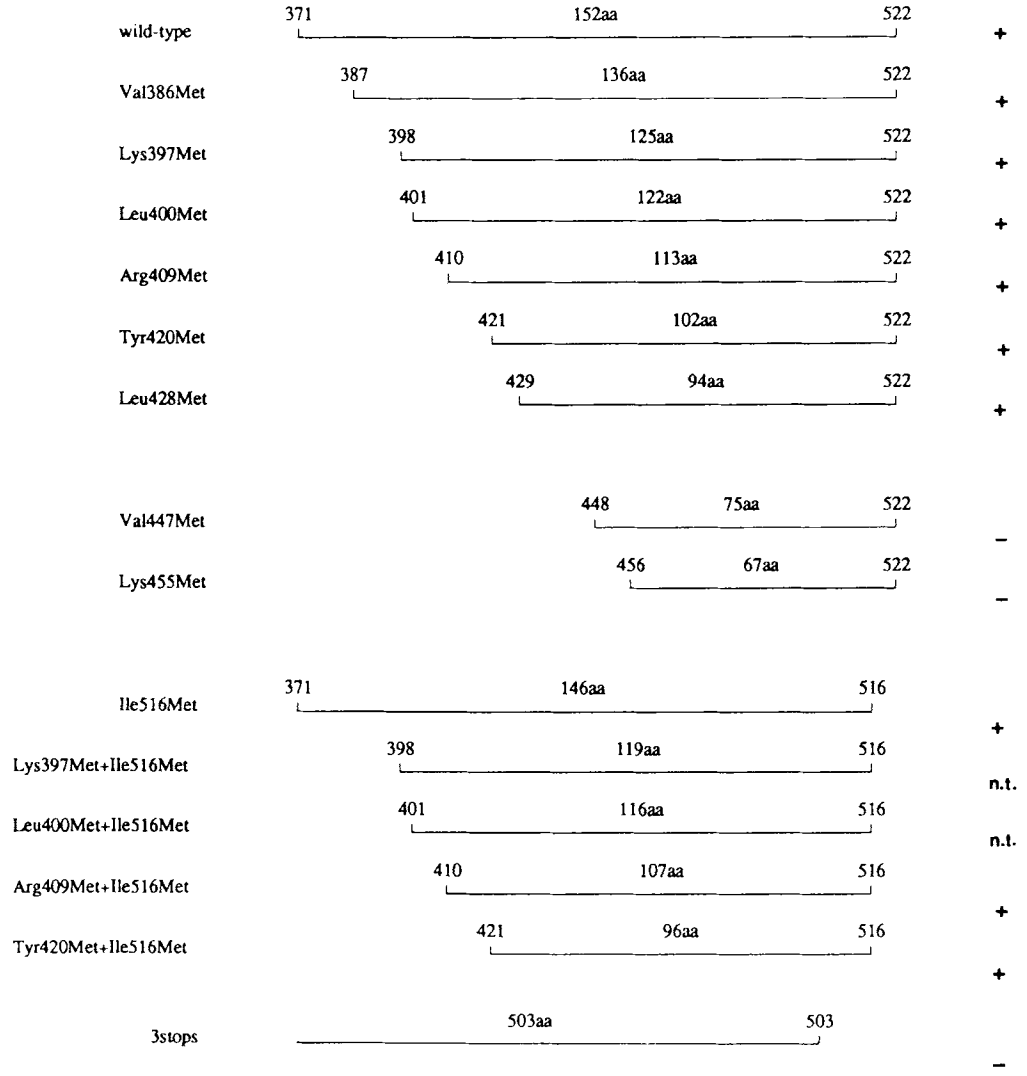


Fig. 7. Diagrammatic representation of the lengths of the COOH-terminal CNBr fragments from various mutants. The lines represent the C-terminal CNBr fragments for the mutants listed on the left. The numbers on both ends of each line indicate the residue numbers; the number in the center of each line indicates the length of the peptide. All of the fragments tested were active in the planar lipid bilayer membranes except those from Val447Met, Lys455Met, and the three stops mutants. All of the single amino acid substitution mutants referred to in this figure produced intact colicin protein molecules which showed full killing activity when assayed with spot tests on sensitive cell. +, conductance induced in planar bilayer membrane is normal. −, conductance induced in planar bilayer membrane is zero. n.t., conductance not tested.

unable to incorporate into the membrane. Within the region of 88 amino acids, which is apparently the maximum length of the channel, there are nine positive and eight negatively charged residues. The turn-on of the channel is most rapid at pH about 3.5, which is at or below the pI of the negatively charged Asp and Glu residues. Thus, there is a large net positive charge to be acted on by the trans-membrane potential.

Implications for a Channel Model

Previous models of the colicin E1 channel have envisioned it as a barrel formed from five or six α-helices, each having 20 amino acids spanning the membrane and two to four residues making the turn at the boundary of the membrane. These models are clearly incompatible with our finding that the channel-forming domain contains no more than 88 amino acids.

The size of the very short peptide able to form a channel in planar lipid bilayers places new restraints on the possible molecular structure of the channel. Since there are not enough amino acids to form 9-Å diameter barrel[10] with multiple α-helices spanning a 30-Å lipid layer, and recent experiments[18] indicate that only one molecule is involved in making the channel, one can consider two other possibilities. First, the lipid layer may have a constriction induced by the protein, so that the thickness of the lipid which must be spanned is significantly less than 30 Å. This idea has been suggested by others,[35,36] who found that membrane-anchoring regions of proteins can be made with fewer than the 20 amino acids needed for an α-helix which spans 30 Å. Another possible struc-

ture is some form of β-sheet wrapped to form a channel; several such structures are now being studied by using the 88 amino acid sequence and will be reported separately.[33] However, the most important consideration is that, given any specific proposal for a molecular structure, it is possible to design mutants to test it. The cysteine mutants we have already made, and additional ones which are currently being made, are designed as attachment sites for sulfhydryl reagents—which can carry nitroxides as election spin labels, moities for fluorescent labels, or groups of various size and charge which would produce alterations in ionic properties of the channel in its open state. Making use of the Cys residues as specifically identifiable attachment sites requires that each molecule to be tested has only one Cys residue, so it was extremely useful that the mutation of Cys505Gly is still active. It has been used in making all of the new Cys mutants indicated in Figure 1.

The experiments reported here clearly raise many new questions. We have cleaved out a very small portion of the colicin protein and found that it is able to attach to phospholipid membranes and form voltage-switchable ion channels. None of the results obtained to date gives any indication concerning the detailed molecular aspects of the events which take place during the transition from the channel-off to the channel-on state. However, one simple hypothesis, consistent with all of the currently available data, is that the protein attaches to the membrane by inserting its 35 amino acid hydrophobic region into the lipid portion of the membrane, and that subsequently the trans-membrane potential acts on the positive charges in the protein to induce a major conformational change to the channel-open state.

Our general model for the action of the C-terminal region of colicin implies that the thermolysin- or trypsin-resistant fragments can take on three different conformations. One is tightly folded, resistant to proteolysis, and very soluble in aqueous solution; a second possible conformation would be found after the peptide has attached to a phospholipid bilayer by virtue of the hydrophobic interaction with residues 474 to 508; the third conformation is the one which forms the open ion channel. Since our current hypothesis for the open-channel conformation is some form of β-sheet wrapped to form a barrel, we are in the process of introducing a sequence of Cys residues in the region necessary for channel formation. Our plan is to use these as attachment sites for reagents sensitive to their local environment, in order to determine which residues are facing out into the lipid and which are in an aqueous environment. The sequence of Cys mutants previously introduced from 398 to 406 was originally designed for this purpose at a time when we thought that the channel-forming region could not be shorter than 129 amino acids. However, although these positions no longer seem to be the ones useful for testing the β-barrel hypothesis, they are useful in helping to explore the conformation of the protein in solution, using electron spin labels, to determine their local environment; these experiments are being done in collaboration with Drs. P. Todd and W.L. Hubbell.

In general, the ability to insert methionine residues for CNBr cleavage and cysteine residues as sulfhydryl attachment sites gives promise of powerful procedures for exploring the conformation, and changes of conformation, of colicin and other membrane proteins.

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