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Topical Review

Studies on the Mechanism of Action of Channel-Forming Colicins Using Artificial Membranes

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Introduction

The demonstration by Schein et al. [54] that colicins A [40] E1, and Ia show voltage-dependent, relatively nonselective ion-permeable channel activity in planar phospholipid bilayer membranes provided a major impetus to the study of the mechanism of action of this group of colicins that also includes K and Ib. Inferences concerning mechanism of action had previously been drawn mainly from physiological and biochemical studies on cells and membranes of *Escherichia coli* [21, 23, 39, 60, 63, 71, 72]. It was known that these colicins kill in a one-hit manner, while inhibiting all macromolecular synthesis and energy-linked functions [21, 27, 39]. It was inferred that the colicins dissipate the bulk phase proton electrochemical potential across the cytoplasmic membrane [21, 23, 63, 71). It has been calculated from the single channel conductance values for colicin E1, $\sim 3 \times 10^6$ ions/channel − sec in 0.1 M salt, that a single colicin molecule can dissipate the cellular membrane potential generated by H^+ pumping [9]. Colicin A has a similar single channel conductance near neutral pH [51] and the values for colicin Ia and Ib are somewhat larger [31, 69]. One can thus view the lethal action of these colicins as occurring through the ability of a single molecule to dissipate the bulk phase membrane potential ($\Delta\Psi$) and thereby de-energize the state of the membrane when $\Delta\Psi$ is the dominant component of the electrochemical potential as defined by Mitchell [45]. An alternative viewpoint, mainly based on studies with energy uncoupled *E. coli* mutants [25, 26], is that these colicins de-energize by disrupting a surface proton current [36].

Subsequent studies on artificial membranes have used both planar and vesicular membranes. Planar membranes are formed from two monolayers by the general method of Montal and Mueller [46]. Briefly, the technique involves forming a bilayer membrane across a small aperture (250 μm diameter for macroscopic conductance, typically 1 nA current; 50 μm diameter for single channel measurements, ~1 pA current) in a partition that separates two aqueous phases. Phospholipid monolayers are spread on the surface of each of the aqueous phases and the two monolayers sequentially raised above the aperture, resulting in the apposition of the monolayers to form a bilayer. This technique avoids or minimizes artifacts arising from the use of organic solvents. Vesicle experiments have been mostly performed with preparations made through a procedure involving prolonged sonication of the lipid suspension, followed by freezing, thawing, and subsequent brief sonication [12, 33]. The resulting vesicle population is of substantially larger size (average diam, ~1000 Å) than that obtained solely by prolonged sonication (average diam, ~250 Å).

The planar membrane technique provides the best method for quantitative measurement of channel conductance, ion selectivity, and channel diameter. In practice, the voltage dependence of colicin action is more readily demonstrable in a planar membrane experiment than with vesicles, as discussed below. The vesicle system is the one of choice for making kinetic measurements and often the better system for measuring biochemical properties of the colicin, simply because one can more readily obtain a useable amount of membrane-bound colicin. These considerations would also apply to many other channel-forming substances. In the case of colicin E1 the dependence of the colicin-membrane interaction on certain parameters such as pH must be measured in the vesicle system, rather than in planar membranes, because the time required to reach steady state in the latter is long. A disadvantage of vesicles for measurements of voltage-dependent colicin-membrane interactions is that such measurements must rely on the use of initially imposed potassium diffusion potentials.

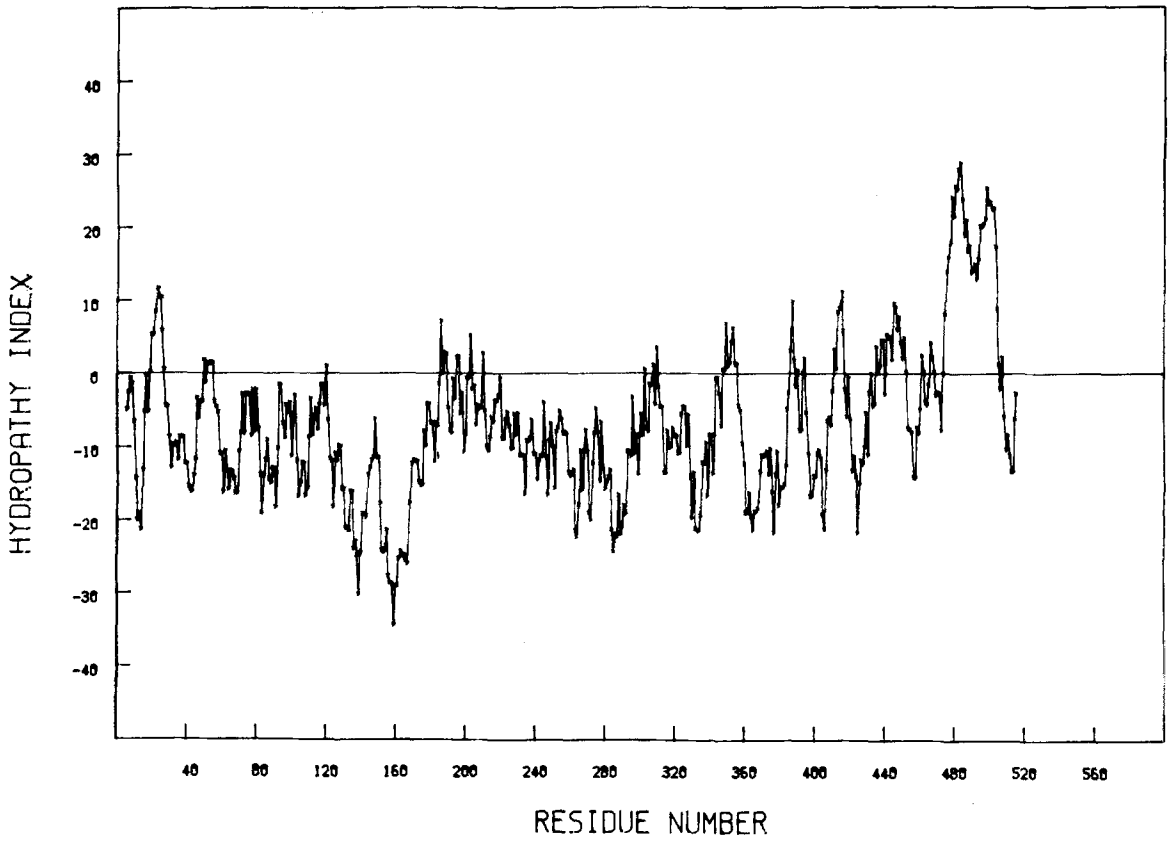

Fig. 1. The hydropathy pattern of colicin E1, using a sliding interval of 11 amino acids according to the algorithm and amino acid free energy transfer values of Kyte and Doolittle [38]. Ordinate values above the baseline correspond to hydrophobic residues

of the polypeptide (Fig. 2). The activity required for channel formation and depolarization of the *E. coli* cytoplasmic membrane has been localized in the C-terminal region, as discussed below, and receptor activity in the central region of the molecule [6]. Except for the question of the function of the N-terminal section of the molecule, left open here, this domain model is analogous to those previously proposed for colicins E2 and E3 [28, 48], and colicin DF13 [14]. The question of whether the channel-forming activity is solely contained in the C-terminal region of the colicin molecule is important in understanding the molecular structure of the channel. The original association of the C-terminal region with the channel was based on the activity of C-terminal peptide fragments added to various cellular and artificial membrane vesicle preparations [11, 49]. Based on this work and subsequent studies on vesicular and planar membranes, three well-defined fragments of colicin E1 have been shown to form channels in artificial membrane systems: (i) an M_r = 20,000 C-terminal trypsin-resistant fragment, starting at residue 336 [11], (ii) a 152-residue CNBr fragment containing residues 371-522 [8], and (iii) an M_r = 18,000 thermolysin fragment of which residue 512 is the C-terminus [49]. These data together indicate that the amino acids comprising the colicin E1 channel must lie between residues 371 and 512. Similarly, an M_r = 20,000 C-terminal bromelain fragment of colicin A has been generated that possesses channel-forming activity [43].

Planar Membrane Experiments on C-terminal Peptide Fragments of Colicins E1 and A

The presence of channel activity in C-terminal peptide fragments of colicin E1 [7, 8], as compared to the absence of activity in a peptide fragment from the central region of the colicin E1 molecule [6], can be seen in a comparison of the macroscopic current caused by addition of colicin E1 (Fig. 3*a*), the C-terminal tryptic fragment (Fig. 3*b*), and a CNBr fragment bordered by methionine residues 230 and 370 (Fig. 3*c*), in the presence of a *trans*-negative membrane potential $\gtrsim -30$ mV. The properties

Table 2. The amino acid sequence of colicin A

```
  1                5                10               15               20
M---P---G---F---N---Y---G---G---K---G---D---G---T---G---W---S---S---E---R---G---S---G---P---E---P---
 26               30               35               40               45
G---G---G---S---H---G---N---S---G---G---H---D---R---G---D---S---S---N---V---G---N---E---S---V---T---
 51               55               60               65               70
V---M---K---P---G---D---S---Y---N---I---P---W---G---K---V---I---I---N---A---A---G---Q---P---T---M---
 76               80               85               90               95
N---G---T---V---M---T---A---D---N---S---S---M---V---P---Y---G---R---G---F---T---R---V---L---N---S---
101              105              110              115              120
L---V---N---N---P---V---S---P---A---G---Q---N---G---G---K---S---P---V---Q---T---A---V---E---N---Y---
126              130              135              140              145
L---M---V---Q---S---G---N---L---P---P---G---Y---W---L---S---N---G---K---V---M---T---E---V---R---E---
151              155              160              165              170
E---R---T---S---G---G---G---G---K---N---G---N---E---R---T---W---T---V---K---V---P---R---E---V---P---
176              180              185              190              195
Q---L---T---A---S---Y---N---E---G---M---R---I---R---Q---E---A---A---D---R---A---R---A---E---A---N---
201              205              210              215              220
A---R---A---L---A---E---E---E---A---R---A---I---A---S---G---K---S---K---A---E---F---D---A---G---K---
226              230              235              240              245
R---V---E---A---A---Q---A---A---I---N---T---A---Q---L---N---V---N---N---L---S---G---A---V---S---A---
251              255              260              265              270
A---N---Q---V---I---T---Q---K---Q---A---E---M---T---P---L---K---N---E---L---A---A---A---N---Q---R---
276              280              285              290              295
V---Q---E---T---L---K---F---I---N---D---P---I---R---S---R---I---H---F---N---M---R---S---G---L---I---
301              305              310              315              320
R---A---Q---H---N---V---D---T---K---Q---N---E---I---N---A---A---V---A---N---R---D---A---L---N---S---
326              330              335              340              345
Q---L---S---Q---A---N---N---I---L---Q---N---A---R---N---E---K---S---A---A---D---A---A---L---S---A---
351              355              360              365              370
A---T---A---Q---R---L---Q---A---E---A---A---L---R---A---A---A---E---A---A---E---K---A---R---Q---R---
376              380              385              390              395
Q---A---E---E---A---E---R---Q---R---Q---A---M---E---V---A---E---K---A---K---D---E---R---E---L---L---
401              405              410              415              420
E---K---T---S---E---L---I---A---G---M---G---D---K---I---G---E---H---L---G---D---K---Y---K---A---I---
426              430              435              440              445
A---K---D---I---A---D---N---I---K---N---F---Q---G---K---T---I---R---S---F---D---D---A---M---A---S---
451              455              460              465              470
L---N---K---I---T---A---N---P---A---M---K---I---N---K---A---D---R---D---A---L---V---N---A---W---K---
476              480              485              490              495
H---V---D---A---Q---D---M---A---N---K---L---G---N---L---S---K---A---F---K---V---A---D---V---V---M---
501              505              510              515              520
K---V---E---K---V---R---E---K---S---I---E---G---Y---E---T---G---N---W---G---P---L---M---L---E---V---
526              530              535              540              545
E---S---W---V---L---S---G---I---A---S---S---V---A---L---G---I---F---S---A---T---L---G---A---Y---A---
551              555              560              565              570
L---S---L---G---V---P---A---I---A---V---G---I---A---G---I---L---L---A---A---V---V---G---A---L---I---
576              580              585              590              595
D---D---K---F---A---D---A---L---N---N---E---I---I---R---P---A---H---
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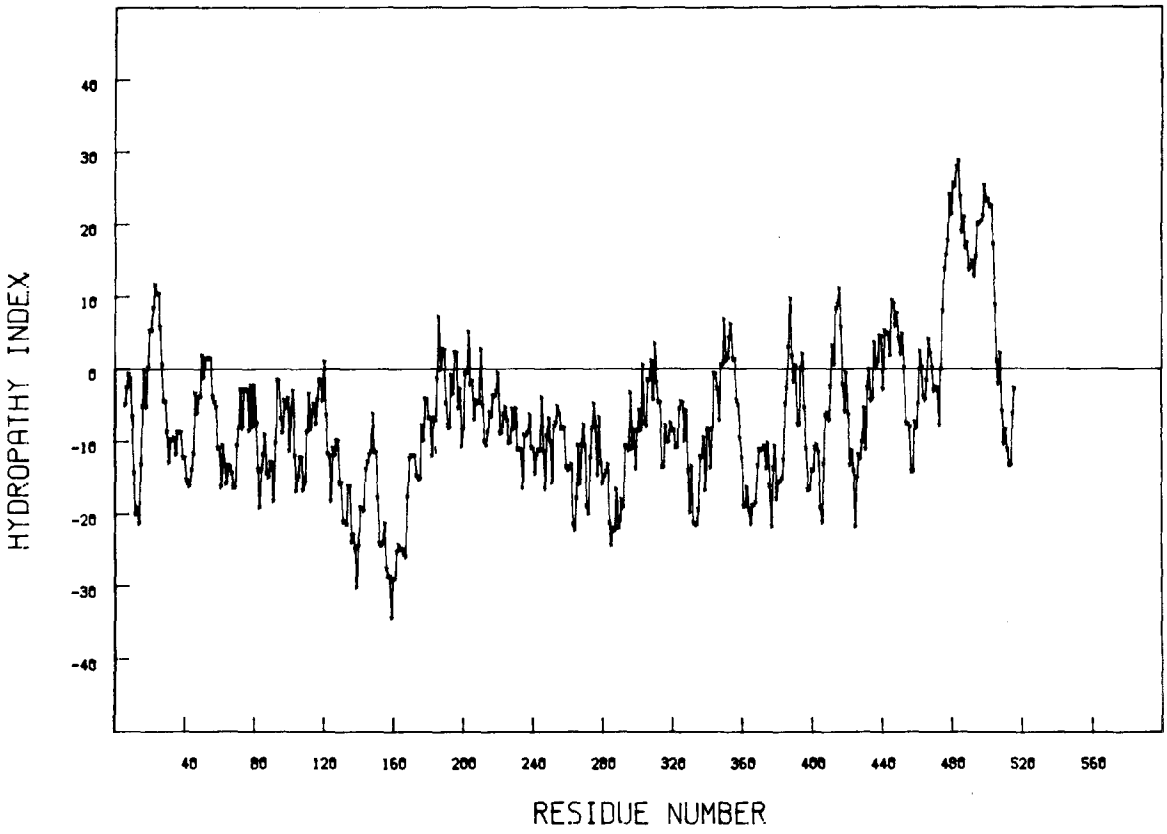
of nonpolar residues in each colicin, the 35-residue sequence 474-508 in colicin E1, and the 49-residue sequence 527-575 in colicin A. The position of this region in the colicin E1 polypeptide is illustrated by the hydropathy pattern, calculated according to the algorithm and free energy values of Kyte and Doolittle [38], shown in Fig. 1. Substitution of the free energy values of Von Heijne and Blomberg [68] in the calculation did not qualitatively alter this pattern. A very similar function can be calculated for colicin A (not shown).

Functional Domains in the Colicin Polypeptide

The amino acid sequence of colicin E1 shown in Table 1 has been organized into a domain structure



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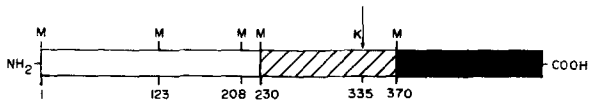

Fig. 2. The current model for the domain structure of colicin E1, showing the position of internal methionines (*M*), and lysine (*K*)-335 just before the site of trypsin cleavage for the C-terminal channel forming tryptic peptide [11]. The smallest C-terminal fragment containing unaltered channel activity according to Cleveland et al. [8] is shown in black extending from the CNBr cleavage site after *met* 370. The smallest peptide isolated with substantial receptor binding activity is symbolized by the cross-hatched section between residue 231 and *met* 370

(i.e., voltage and pH dependence, single channel conductance, selectivity, rectification) of the C-terminal fragment were hardly distinguishable from those of the parental molecule [7]. Single channel conductance measurements of colicin E1 and the C-terminal tryptic fragment are shown in Fig. 4*A* and *B*, and multievent histograms defining the average single channel conductance are shown in Fig. 5*A* and *B*. The average single channel conductance measured in asolectin membranes was found from Fig. 5*A* and *B* to be 20.9 ± 3.9 pS and 19.1 ± 2.9 pS, respectively, for colicin E1 and the C-terminal tryptic fragment at pH 6.0. A similar value at pH 4.5, where colicin and fragment activity are much greater than at pH 6.0, was found for the smallest C-terminal fragment thus far isolated with unimpaired channel activity, the 152 residue fragment made by cyanogen bromide cleavage at methionine 370 [8]. The "turnoff" time of the current after imposition of a *trans*-positive potential is much shorter at pH 4.0 (Fig. 6) than pH 6.0 (Fig. 3*a* and *b*). The "turnoff" time observed in Fig. 3*c* is unusually short for an experiment at pH 6.0.

The isolation of a C-terminal peptide of colicin A with channel activity, after proteolytic treatment of the parental molecule with bromelain [43], provided further confirmation of the general applicability to channel-forming colicin of the domain model shown above in Fig. 2. Furthermore, both colicin A and its bromelain fragment required *trans*-negative potentials to form conducting channels. Both molecules exhibited similar single channel conductances as a function of pH and applied voltage [43, 51], although the on-off behavior of the fragment channels as a function of potential showed a larger hysteresis than that of the colicin. The single channel conductance varied from 5 to ~15 pS with imposed *trans*-membrane potentials of 100 and −100 mV, respectively, in 1 M KCl at pH 7.0, and decreased from ~15 to 5 pS at −100 mV as the pH was lowered from 7.0 to 5.0 [51]. This strong dependence of

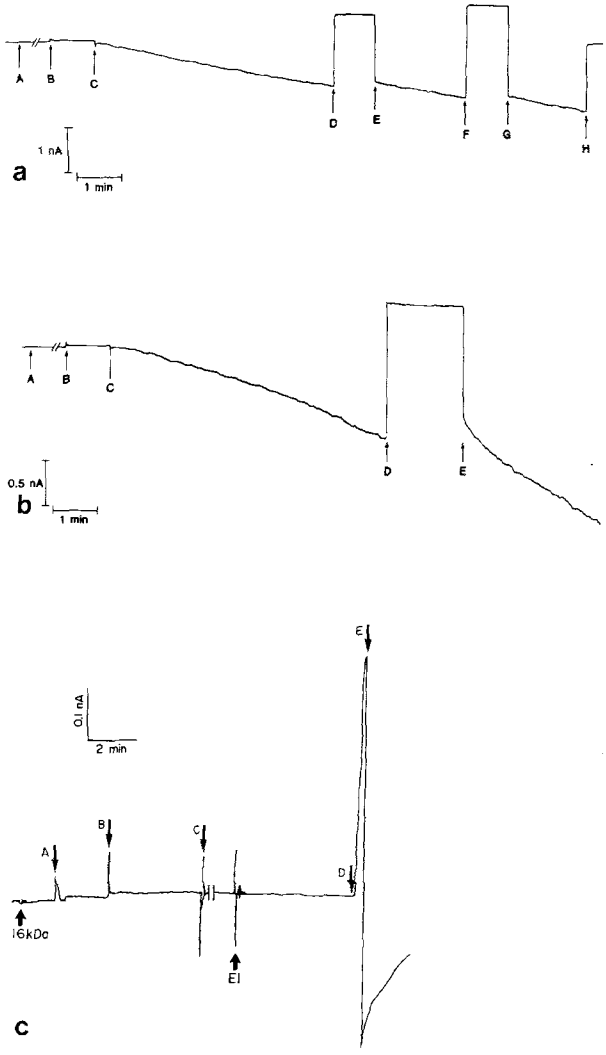

Fig. 3. Macroscopic conductance properties induced by addition (~0.5 μg/ml) of (*a*) colicin E1, (*b*) the C-terminal tryptic fragment, and (*c*) the 140-residue cyanogen bromide receptor-binding fragment, the fragment corresponding to the hatched region in Fig. 2, to a voltage-clamped planar membrane. *Trans*-negative potentials correspond to downward deflections in *a* and *b*, and an upward deflection in *c*. (*a*): Colicin E1 was added to one side (*cis*) of the membrane (*A*). No increase in current was observed with the voltage of the *trans* relative to the *cis* side, initially clamped at +80 mV (*B*). When the voltage was switched to −80 mV (*C*), the current increased steadily. Switching the membrane potential to +80 mV (*D* and *F*) resulted in cessation of the conductance increase, and switching both to −80 mV (*E* and *G*) in its resumption. Switching to 0 mV (*H*) redefined the baseline [7]. (*b*): C-terminal tryptic fragment was added to one side (*cis*) of the membrane (*A*). Voltage was applied to the membrane as follows: At *B*, +50 mV, at *C*, −50 mV; at *D*, +50 mV; at *E*, −50 mV [7]. (*c*): 16 kDa CNBr fragment was added to the *cis* side. Voltages were applied as follows: *A*, −50 mV; *B*, −80 mV; *C*, −90 mV. 11 min after adding the fragment, colicin E1 was added to the *cis* side. Voltages of −70 mV (0) and +70 mV (*E*) were applied [6]. Symmetrical solutions of 1 M NaCl, 10 mM MES buffer, 3 mM $CaCl_2$, 0.1 mM EDTA, pH 6.0, bathed the membrane. Reprinted with permission of *J. Biol. Chem.*

the single channel conductance is not observed for colicin E1 as a function of voltage [7, 8]. The ratio of the transport number of Cl^- to K^+ in planar membranes made of neutral diphytanoylphosphatidylcholine lipid changed from about one at pH 7.0 to about five at pH 4.5 (S. Slatin, L. Raymond and A. Finkelstein, *personal communication*). Thus, the colicin E1 channel was more anion selective at low pH.

The Question of the Potential Dependence of Colicin Action on Membrane Vesicles

The requirement of a membrane potential for colicin action *in vitro* in planar membranes provided an explanation [54] for the requirement of an energized cellular membrane for colicin action [30]. One might then initially assume that the dependence of colicin action on the sign and magnitude of the membrane

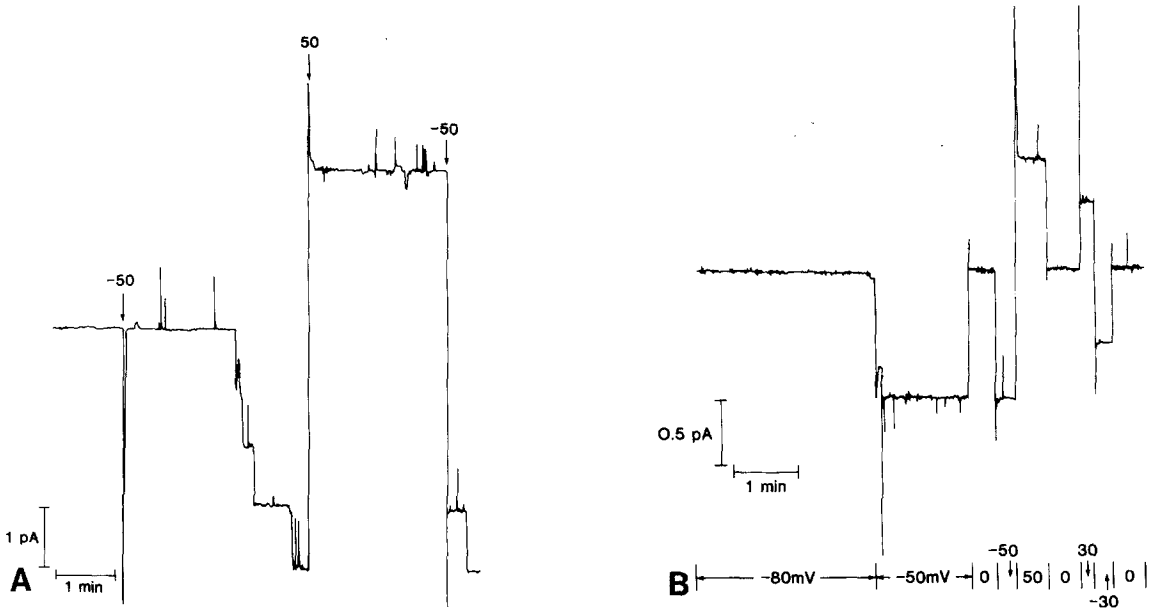

Fig. 4. Single channel recordings of (*A*) colicin E1 and (*B*) the C-terminal tryptic peptide. Colicin or fragment was added to one side (*cis*) of the membrane (*A*): The opening of single colicin and fragment channels was observed after the membrane was clamped at −50 mV. When the voltage was switched to +50 mV, a single channel quickly turned off, a relatively rare event. The remaining three colicin channels stayed open while the membrane was clamped at +50 mV. When the membrane potential was switched back to −50 mV, three channels were initially present and then a fourth channel opened. (*B*): A +80-mV potential did not cause opening of channels. Channel opening was observed when the potential was changed to −80 mV, which was then immediately changed to −50 mV in this trace, and then to the other voltages as indicated. The bathing solution for the membrane was as described for Fig. 3. Reprinted with permission from *J. Biol. Chem.* [7]

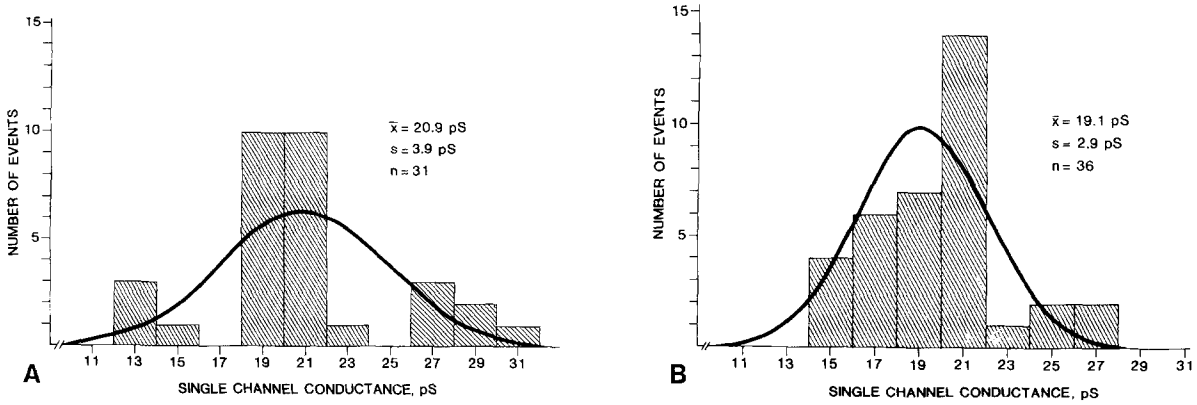

Fig. 5. Histograms of the frequency distribution for values of single channel conductance. The indicated single channel conductances were measured at −50 mV. (*A*): Colicin E1. (*B*): C-Terminal tryptic fragment. Solid lines represent Gaussian distributions normalized to the number of single channel measurements. The means and standard deviations are indicated. Bathing solution as in Fig. 3

potential in planar membranes should be readily observable in artificial membrane vesicles. The lack of such an observed dependence [34, 64] might lead, conversely, to the conclusion that the voltage dependence observed in planar membranes is not physiologically relevant. It is important to state that the average diameter of the vesicle populations on which colicin activity was tested and found not to depend on voltage was approximately 0.1 μm [12, 34, 64]. The presence of a voltage dependence has been found, however, using larger vesicles with ~0.5 μm diameter formed by a calcium-induced fusion technique [12, 50] and monitoring release of trapped Cl^- [12 and Fig. 7]. This potential dependence, showing a fivefold change in the rate of colicin E1 action for a change of 160 mV in imposed potential, was much smaller than that seen in planar membranes, where an *e*-fold change of current for each 3.7-mV change in potential was observed for colicin A [54]. The explanation for the lack of potential dependence in the 0.1-μm diameter vesicles, and the weak effect in the larger 0.5-μm diameter vesicles (Fig. 7), very likely resides in the small membrane capacitance and volume capacity of the vesicles. It can be calculated, as in ref. 9, that a 100-mV diffusion potential in the 0.1-μm vesicles would be dissipated by movement of only about 300 charges across the vesicle surface. Depolarization of the 0.5-μm diameter vesicles would require a larger number of translocated charges, $25 \times (300) = 7500$ charges. The insertion of a single colicin channel into a vesicle of either size group would therefore completely depolarize that vesicle. In fact, if binding and insertion of a single colicin induced a permeability increase that was only a small fraction (10^{-4} for the small vesicles and 2.5×10^{-3} for the large ones) of the single channel conductance, the membrane potential would be dissipated in one second. This leads to the hypothesis that the lack of a voltage dependence seen in the smaller vesicles and the small dependence observed in the larger 0.5-μm diameter vesicles could be caused by depolarization during the initial voltage-independent binding and insertion events that precede channel formation. Recent data on the nature of the voltage-independent binding and insertion of colicin A into phospholipid monolayers lend support to this view. By measuring the increase in surface area that results from colicin A associating with a monolayer film at constant surface pressure, it was calculated that at pH 5 the apparent molecular area occupied by the protein was 2000 $Å^2$ at a surface pressure of 20 dyne/cm [52]. This is at least as large as the cross section that one would calculate for the colicin channel fully inserted into the membrane. This voltage-independent colicin A binding thus caused a large perturbation of the membrane surface that could readily result in a small ion leak sufficient to cause depolarization.

The fact that a large fraction of the colicin A molecule penetrates the monolayer also seems im-

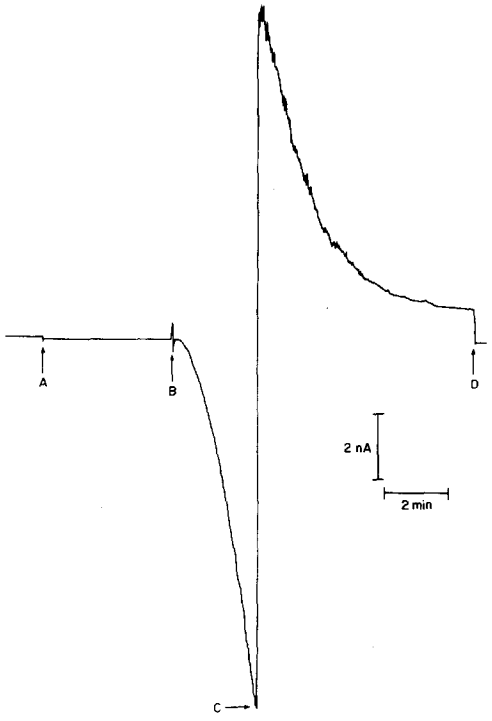

Fig. 6. Macroscopic current measurement of the C-terminal tryptic fragment at pH 4.0. Fragment added (*B*) to membranes clamped at −20 mV (*A*, *trans* side negative). Increase of current is seen while potential is maintained at −20 mV and rapid decay to baseline observed when the voltage is switched (*C*) to +20 mV. Voltage is set to zero at (*D*)

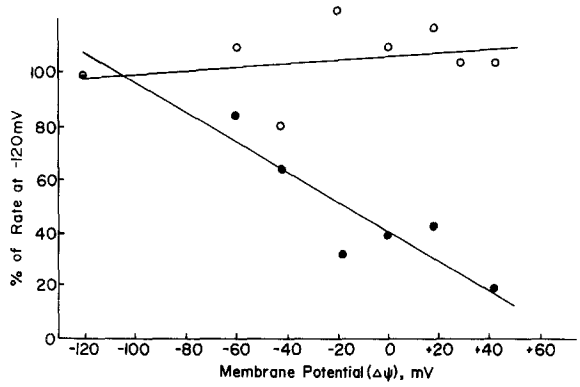

Fig. 7. The dependence of the rate of colicin-induced Cl^- efflux on the membrane potential, $\Delta\Psi$, for large (0.5 μm diameter) vesicles made by a fusion technique [12], and smaller (0.1 μm diameter) vesicles made by freeze-thaw. (○) freeze-thaw vesicles; (●) fused vesicles; pH, 4.0. Details described in ref. 12. Reprinted with permission from *J. Biol. Chem.* [12]

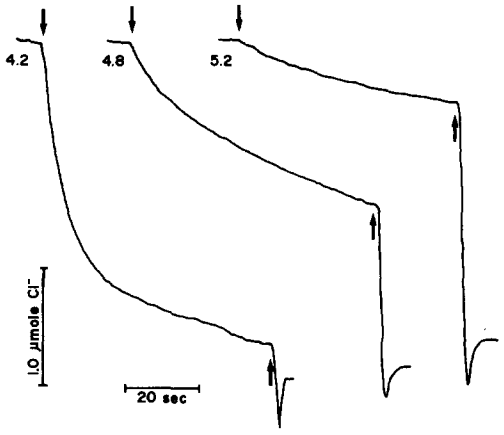

Fig. 8. Chloride efflux induced by addition by colicin E1 (2 μg/ml) to a population of fused asolectin membrane vesicles. K^+-diffusion potential, −60 mV. (↓) and (↑) indicate addition of colicin and 0.25% Triton-X-100. Ambient pH values of 4.2, 4.8, and 5.2, respectively, are indicated on traces. Reprinted with permission from *J. Biol. Chem.* [12]

portant in understanding the mechanism by which the colicin channel is gated or regulated by the membrane potential. Since at low pH the bulk of the colicin molecule is fully inserted into at least one of the monolayers in the absence of a potential, the potential must change the conformation of the inserted protein, or insert only a small additional part of the protein into the membrane. A *caveat* in this reasoning is the question of whether the behavior of the colicin in monolayers will apply to bilayers. A different view of the mechanism of voltage-dependent gating, involving a voltage-dependent insertion of a major portion of the colicin polypeptide, was proposed by Cleveland et al. [8].

pH Dependence of Colicin Action

Acidic pH caused an increase in the macroscopic conductance response of colicin E1 [7]. This effect resembles the pH dependence *in vitro* of the action of diphtheria [16, 17, 32] and tetanus toxins [3], and suggests the possibility of a general requirement of low pH for the *in vitro* insertion of toxin-like molecules or toxins and viruses [42] into membranes. A possible molecular explanation for the acidic pH requirement will be discussed below.

The detailed pH dependence of colicin E1 action on planar membranes is difficult to measure because the time required, after colicin addition, for attainment of a steady-state current is very long (Fig. 3*a* and *b*) at suboptimal pH values. The pH dependence of the action of colicin E1 in causing chloride efflux from vesicles loaded with chloride is shown in Fig. 8. The rate of Cl^- efflux is plotted as a function of pH in Fig. 9*A* and *B*. The effective pK values for Cl^- efflux caused by addition of colicin E1 and its C-terminal tryptic peptide are 4.6 and ≤3.8, respectively. The 0.8-pH unit difference presumably arises from a different environment of the critical acidic group(s) of the peptide relative to the parental molecule. These pH values could be shifted downward by the increase in anion selectivity of the channel at low pH [61] and the use of Cl^- efflux to measure activity. The activity of colicin E1, unlike that of the fragment, showed a decrease below pH 4.0 (Fig. 9*A*). This may be due to denaturing effects on the N-terminal two-thirds of the colicin molecule, with a consequent effect on the channel-forming domain.

Studies on the interaction of [^{35}S] colicin A with tritiated asolectin vesicles and phospholipid monolayers showed a strong effect of pH on the binding and strength of interaction [52]. The binding of ^{35}S-labeled colicin A to the ^{3}H vesicles was much stronger at pH 5 relative to 7. This effect may be similar to the increase in binding of diphtheria toxin to Triton X-100 detergent at low pH [53]. Additional information on the colicin A interaction with lipid was obtained by measurement of the change in surface pressure of a phospholipid monolayer caused by addition of colicin A [52]. The effect on surface pressure again increased as the pH was lowered, and titrated with an effective pK of the colicin A molecule of about 5.5. This pK was similar to that which appeared to govern the pH dependence of colicin A single channel conductance [51], referred to above. Thus, there is a general agreement in these studies on colicins A and E1 that low pH and protonation of a specific amino acid residue(s) are required for effective binding or insertion of the colicin to or into the membrane.

Regarding the physiological significance of the requirement of an acidic pH for action of colicins E1 and A *in vitro*, we had previously put forward the suggestion that the pH of the periplasmic space, through which the colicin must pass, might be very acidic [9]. This possibility now seems less likely since the sensitivity of the cells to colicin is unchanged when incubated in 0.1 M phosphate at an external pH of 7 [10]. One would suspect that the phosphate ion, which is accessible to the periplasm via the porins, would effectively buffer the periplasmic space. It appears more likely that *in vivo* a certain residue(s) in the colicin polypeptide must be protonated in order that the colicin molecule be able to insert into the membrane. An environment causing an increase in the effective pK of these residues in the cell could be conferred by the appropriate

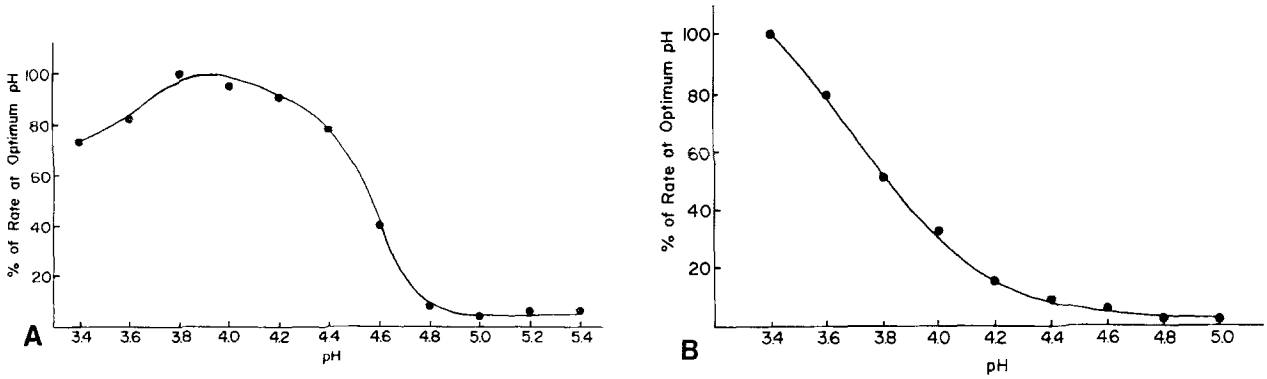

Fig. 9. pH dependence of Cl^- efflux from fused vesicles caused by addition of (*A*) colicin E1 (0.5 μg/ml) or (*B*) C-terminal tryptic fragment (0.3 μg/ml). Data points represent the average of two experiments. Initial K^+ diffusion potential set at −60 mV, with valinomycin (5×10^{-8} M) added 20 sec before addition of protein. Reprinted with permission from *J. Biol. Chem.* [12]

colicin conformation imposed by the cell receptor or membrane. Alternatively, the acidic pH requirement could explain the low absolute activity of colicin E1 *in vivo* [9]. The pK values associated *in vitro* with this protonation, 3.8 and 4.6 for C-terminal fragment and colicin E1, and 5.5 for colicin A, suggest that the critical residues could be carboxylic acid and/or histidine residues. For colicin E1, the first possibility now appears to be the more likely because (i) pK values near 4.0 are more often associated with carboxyl groups in proteins and (ii) modification with the histidine-labeling reagent, diethylpyrocarbonate, of the two histidine residues in the C-terminal tryptic peptide does not alter the pH dependence of colicin fragment-induced chloride efflux from vesicles or the macroscopic conductance of the colicin fragment in planar membranes (L.J. Bishop, W.A. Cramer and F.S. Cohen, *unpublished*). Relevant to a role of a carboxylic acid residue in accounting for the pH dependence is a description of membrane spanning protein domains that might contribute to the colicin channel (Fig. 10, discussed below), in which one charged residue, glutamic acid (*glu* 468) in helix IV, residues in an otherwise hydrophobic environment.

Other observations related to the pH dependence of colicin action that have been obtained recently enough that full interpretation is not yet available are: (i) As mentioned above, the "turn-off" time in planar membranes is strongly pH-dependent. This is the characteristic time required for decay of the current to the baseline level after the voltage is switched from the highly conductive *trans*-negative state to a *trans*-positive state. This is illustrated by comparison of Figs. 6 and 3*A* and *B*, and of refs. 7 and 8. The times decrease rapidly with decreasing pH. At pH 3.5, it has been found recently (S. Slatin et al., *personal communication*) that the turn-off times are on the order of msec for sufficiently large voltages (~100 mV). (ii) Another important consequence of studies at pH 3.5 is that the macroscopic current for colicin E1 was found to reach a steady state. It was then possible to measure the current as a function of the concentration of added colicin E1 and obtain a dose-response curve which was linear (S. Slatin et al., *personal communication*), as it was for colicin A [54]. This indicates that a single colicin molecule is competent to make a channel, consistent with the classical view of these colicins as single-hit molecules [27, 39].

Size of the Colicin E1 Channel

The size of the channel has been estimated in two studies, one in vesicles made of DMPC lipid below the T_m of the lipid [66], and one in planar membranes made of asolectin [61]. The estimated channel diameters from the two studies are somewhat different, 6–6.5 and 8.0 Å, respectively. The channel size was estimated in DMPC vesicles containing incorporated colicin E1 by the rate of efflux, after dilution, of solutes of different size. An approximate cut-off was extrapolated to a solute size near that of glycerol (hydrated ionic radius determined by viscometry, 3.1 Å [56]). The planar membrane measurements, based on determination of the reversal potential generated by salt gradients across the membrane showed that the cation glucosamine (unhydrated ionic radius = 4 Å [S. Slatin et al., *personal communication*]) and the anion glucuro-

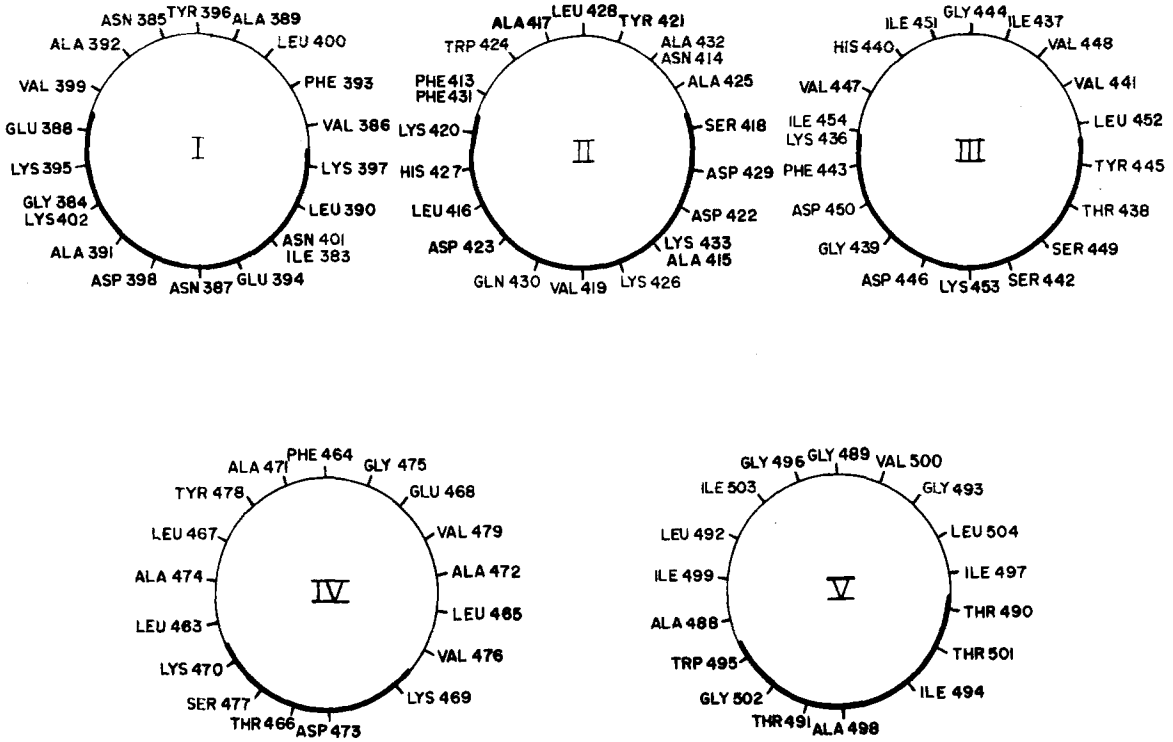

Fig. 10. Five potentially amphipathic helices which may comprise the colicin E1 channel. The residues of each helical segment are plotted as a wheel in a two dimensional projection of the helix [55]. The hydrophobic and hydrophilic faces of each helix, determined as described in the text, are indicated, respectively, by bold and thin arcs

nate (unhydrated ionic radius = 4 Å [S. Slatin, et al., *personal communication*]) are impermeant, whereas the smaller tetraethylammonium ion was slightly permeant. Extrapolation in a double reciprocal plot to zero transfer number yielded a limiting solute size of about 8.0 Å ([61], S. Slatin, et al., *personal communication*). Studies on colicins A and Ia also indicate a size limitation to flow, demonstrated by the slow efflux from loaded vesicles of sucrose [34, 64], sulfate [34], and glucose-6-phosphate [64].

A Model for the Channel

As yet we are not aware of molecular model building studies on the colicin channel for colicin A, Ia, or K. Therefore, the following discussion is focused on colicin E1. The first quantitative model for the channel was that of Guy [24]. It required a dimeric model, each monomer containing nine helices, starting at residue 355 in each polypeptide. Although the model has many strong points, it appears at present to be incorrect in some details because the C-terminal CNBr fragment [8], starting at residue 371, was shown to have full channel-forming activity. This model also included residues beyond residue 512. Thus, two of the nine helical segments of each monomer are comprised of amino acids outside the range of residues 371-512 and cannot therefore be essential for channel formation. The model required demonstration of (i) "two hit" kinetic data *in vivo* and *in vitro* for the action of native colicin as well as the C-terminal fragments (*see* discussion in ref. 9), or (ii) a naturally occurring dimer or oligomer of the colicin and all the active fragments. Neither i nor ii has been demonstrated. In addition, the dose-response curve of the macroscopic conductance of colicin A [54], and of colicin E1 measured at low pH (S. Slatin et al., *personal communication*), has been shown to have a slope of one, implying that the channel is formed by a single molecule.

Thus, the bulk of the evidence at present indicates that the depolarizing channel formed in the inner membrane of the initially energized *E. coli* must be formed by a single polypeptide derived from the C-terminal end of the colicin molecule. This is in contrast to oligomeric channels such as *Staphylococcus aureus* α-toxin [2], melittin [65], and the proteolipid of the H^+-translocating ATPase [57]. The following considerations must be applied in building a model from the single polypeptide for the colicin E1 channel: (i) Its diameter is 6–8 Å

([66], S. Slatin et al., *personal communication*). (ii) The smallest C-terminal fragment in which full activity has been obtained is the 152-residue CNBr fragment. Since it has been shown that the last 10 residues of the E1 molecule are not essential for activity [49], it appears that the channel can be formed from 142 residues, 371-512, of the polypeptide. (iii) Using recombinant DNA techniques, a truncated colicin E1 molecule was constructed from which eighteen C-terminal amino acids have been removed and the following six (E-D-D-R-A-S) added (V.L. Davidson, H. Zalkin and W.A. Cramer, *unpublished*). This molecule was made by cutting the E1 structural gene at an unique *Eco* R1 site and splicing it into pBR322 at this site. As this colicin was inactive both *in vivo* and *in vitro*, we infer that the critical limit to length at the C-terminal end of the protein lies between residues 504 and 512. (iv) There is a paucity of secondary structure data for membrane proteins in general, and without crystal structure determination, existing methods may not be sufficiently quantitative to distinguish between different models. Circular dichroism measurements of the secondary structure content of the M_r = 20,000 C-terminal tryptic fragment of colicin E1 in the detergent octylglucoside indicated an α-helical content at pH 3.5 of 55–60%, or approximately 95–110 residues in this conformation (K.R. Brunden, Y. Uratani and W.A. Cramer, *in preparation*). (v) Qualitative estimates indicate that construction of a 6–8 Å channel with α-helices, whose center-to-center distance is 10–12 Å, depending on side-chain interactions, requires five membrane-spanning helices (V.L. Davidson and W.A. Cramer, *unpublished*). (vi) The actual number of membrane-spanning domains seems to be an even number, and would therefore be six, for the following reasons: In planar membrane experiments, addition of large amounts of carboxypeptidase Y (0.5 mg/ml) to the *cis*, but not the *trans*, chamber causes a large decrease in the macroscopic current associated with colicin E1 after a transient increase. This suggests that the C-terminus of the colicin resides on the *cis* side of the bilayer [8]. Because colicin E1 has activity *in vitro* similar to that of the C-terminal fragments, the other end of the channel-forming polypeptide domain must also emerge on the *cis* side, as it is connected to the relatively polar remaining two-thirds of the colicin molecule. The latter segment is probably not translocated across the membrane.

Even if the helical content of the colicin channel embedded in the membrane turns out to be greater than the preliminary estimates cited above, the problem of building a model for the colicin channel appears formidable. This is illustrated by comparing the problem of building the six membrane spanning units of the colicin channel from 142 residues to that of the model for bacteriorhodopsin that utilized seven membrane-spanning domains derived from 248 residues [19]. It is, of course, an assumption that the membrane-spanning units forming the channel are α-helices. It has been argued that α-helices are energetically the favored state for membrane-spanning domains, based partly on experimental data for the bacteriorhodopsin structure, indicating that it consisted of seven membrane-spanning α-helices [20]. However, a significant β-sheet contribution to this structure has recently been proposed [29], and a predominantly β-structure has been proposed for the porin channel (M. Garavito and J.P. Rosenbusch, *personal communication*).

We propose a channel made of amphipathic helices to describe at least part of the structure of the lipid-embedded water-filled channel. A similar structure has been proposed as a model for the alamethicin channel [22]. Amphipathic helices are defined by the separation of nonpolar and polar faces as one looks down the helix axis. Such a helix would have its nonpolar face oriented toward the lipid and the polar face toward the interior of the channel. Amphipathic helices were originally proposed to explain the interactions of apolipoproteins with the plasma membrane [59]. More recently, the sequences of three membrane active toxins, melittin [13, 15], cecropin A [44, 62], and diphtheria [35], have been shown to contain potentially amphipathic helical segments. Starting with residue 383 which occurs after a double *lys* in the colicin E1 sequence, five possible amphipathic helices have been defined using the algorithm of Segrest and Feldmann [58]: Helix I, *ile* 383-*lys* 405; helix II, *ala* 415-*lys* 433; helix III, *lys* 436- *ile* 454; helix IV, *leu* 463- *val* 479; helix V, *ala* 488- *leu* 504. The residues of each segment are plotted as helical wheels (ref. 55, Fig. 10). The center of the hydrophobic or nonpolar face of these wheels coincides in each case with a maximum value of the hydrophobic moment calculated according to the method of Eisenberg et al. [18]. The separation of hydrophobic and hydrophilic faces shown in Fig. 10 was determined by maximizing for each helix the difference in hydropathy index [38] of the two faces. The average length of these helices is 19 residues, ranging in length from 17 (helix IV) to 23 (helix I) residues. This corresponds to an average helix length of 19 · (1.5 Å/α helical residue) = 28.5 Å. The number of α-helical residues then adds up to 96, within the prediction mentioned above for the C-terminal tryptic fragment made by circular dichroism analysis (K.R. Brunden et al., *in preparation*). The identity of a

possible sixth spanning domain, discussed above, is not known at present. A qualification that one must keep in mind, with respect to the diagrams shown in Fig. 10, is that these are planar representations of helices that are idealized in the sense that the adjacent side chains are spaced exactly 100° apart and perpendicular to the helix axis. It can be seen using physical models (V.L. Davidson and W.A. Cramer, *unpublished*) that this angle can actually vary significantly because of rotational freedom of the constituent *R* groups.

There is one violation of the amphipathic distribution of residues in the above helices, the presence of one charged residue, *glu* 468, found in the nonpolar region of helix IV. Insertion of this uncompensated charge into the lipophilic phase would involve a large free energy barrier [68]. We suggest that it is the protonation of this particular acidic charged residue that is responsible for the greater efficiency of colicin and fragment action *in vitro* at pH values ≤ 4.0. We propose that *in vivo* the effective pK of this residue must be shifted to much more positive values by the membrane protein environment so that it is readily protonated at the effective surrounding pH. From Fig. 10, *his* 440 occurring in helix III appears to be another possible charged residue positioned in a hydrophobic surrounding. Since most transfer free energy values for amino acid side chains presume a neutral pH environment, histidine is usually considered an uncharged residue [38]. However, for proteins requiring an acidic pH for activity, such as colicins E1 and A, and diphtheria and tetanus toxins, histidine should be positively charged. Although *his* 440 resides in the hydrophobic arc of helix III, a physical model of the colicin E1 channel shows that the ring of *his* 440 can be rotated about the β carbon so that it is close to the polar interface of helix III (V.L. Davidson and W.A. Cramer, *unpublished*). To the extent that *his* 440 does lie in a nonpolar environment, it is known that the contribution of charge for a histidine residue to its free energy of transfer is small relative to the contribution of charge for *glu, asp, lys,* and *arg* residues [68]. For these two reasons, we do not consider the position of this residue in helix III to be inconsistent with the amphipathic helix model of Fig. 10.

Another preliminary model has been described by Cleveland et al. [8], who propose that spontaneous attachment of the colicin molecule to the membrane occurs through an α-helical hairpin centered on the 35-residue hydrophobic sequence of amino acids between residues 474 and 508, near the C-terminus (Table 1, Fig. 1). This proposal would presumably apply also to the similar 49-residue hydrophobic segment in colicin A (Table 2, ref. 47). The model also proposes that upon imposition of a *trans*-negative potential, additional segments of the protein insert into the membrane forming either three or four additional transmembrane helices. The latter aspect of the model implies the existence of a large energy barrier for insertion of the additional helices by the potential, and would not readily explain the large surface area occupied by colicin A in monolayers in the absence of a potential [52].

Colicin Translocation Through The Outer Membrane

A hypothetical description for the pathway and action of the channel-forming colicins from solution to the membrane would be as follows: In the extracellular medium, the hydrophobic core of the colicin protein contains the hydrophobic C-terminal section of the molecule. The colicin binds to the receptor in the outer membrane through the central region of the colicin molecule, perhaps using the N-terminal third of the colicin molecule to achieve effective binding and translocation. As part of the translocation process through the outer membrane, the C-terminal region is unmasked and inserted into the periplasm, possibly through the Bayer junctions [1]. We propose that the C-terminal end of the colicin may be involved in the initial reversible binding to the inner membrane. The C-terminal end of the colicin E1 molecule, starting with the charged *lys* 512, proceeding to the charged sequence K-X-K-D between residues 512-509, and then to the nonpolar sequence between residues 508-474, bears a sequence analogy to the signal peptide region used at the N-terminus for protein secretion [67]. This C-terminal region could then be used for initial binding. After the initial attachment, the amphipathic helices would be oriented along the membrane surface, with their polar sides exposed to the aqueous phase and nonpolar sides imbedded in the hydrocarbon layer. The membrane spanning segments within a colicin molecule could then form a cluster and cooperatively insert, thereby shielding the polar faces on the inside of the channel.

An obvious possibility in this proposal is that the C-terminal channel-forming domain might be freed by *in vivo* proteolysis from the rest of the colicin molecule after translocation through the outer membrane receptor. This would allow the C-terminal domain to form the channel in the inner membrane without any constraint from the rest of the polypeptide attached to the outer membrane. The consensus in the literature at present, however, is that the colicin does not undergo proteolysis as part of its mechanism of action [4, 5]. This would

also be the implication of the trypsin rescue experiment in which trypsin added to the external medium outside the cells could restore active transport and viability to cells in which transport had already been inhibited by colicin E1 [10].

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