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Gating of a Voltage-Dependent Channel (Colicin E1) in Planar Lipid Bilayers: Translocation of Regions Outside the Channel-Forming Domain

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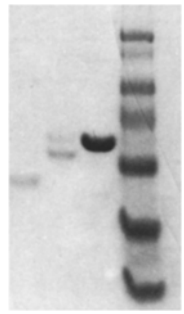
Summary. C-terminal fragments of colicin E1, ranging in mol wt from 14.5 to 20 kD, form channels with voltage dependence and ion selectivity qualitatively similar to those of whole E1, placing an upper limit on the channel-forming domain. Under certain conditions, however, the gating kinetics and ion selectivity of channels formed by these different E1 peptides can be distinguished. The differences in channel behavior appear to be correlated with peptide length. Enzymatic digestion with trypsin of membrane-bound E1 peptides converts channel behavior of longer peptides to that characteristic of channels formed by shorter fragments. Apparently trypsin removes segments of protein N-terminal to the channel-forming region, since gating behavior of the shortest fragment is little affected by the enzyme. The success of this conversion depends on the side of the membrane to which trypsin is added and on the state, open or closed, of the channel. Trypsin modifies only closed channels from the *cis* side (the side to which protein has been added) and only open channels from the *trans* side. These results suggest that regions outside the channel-forming domain affect ion selectivity and gating, and they also provide evidence that large protein segments outside the channel-forming domain are translocated across the membrane with channel gating.

Key Words colicin E1 fragments · lipid bilayers · channels · voltage gating · protein translocation

Introduction

The colicin E1 molecule can be divided into regions of functional specialization. C-terminal fragments (*see* Fig. 1), generated by cyanogen bromide cleavage (CNBr) or enzymatic cleavage, contain the channel-forming activity (Dankert et al., 1982; Ohno-Iwashita & Imahori, 1982; Bullock, Cohen, Dankert & Cramer, 1983; Cleveland, Slatin, Finkelstein & Levinthal, 1983), whereas regions that lie N-terminal to these function in receptor binding (Ohno-Iwashita & Imahori, 1982; Brunden, Cramer & Cohen 1984) and translocation of part or all of the molecule from the outer to the inner bacterial membrane (Ohno-Iwashita & Imahori, 1982). Channels formed by three of these C-terminal fragments (generated by cleavage with trypsin, thermolysin, and CNBr) have been studied in planar bilayer membranes and found to be very similar to those formed by whole colicin E1 in voltage dependence, selectivity, and single-channel conductance. The fragments were characterized, however, under a narrow set of conditions. The 178-amino acid thermolysin fragment, herein designated ThCF, and the 152-amino acid CNBr fragment, designated CNBr, were tested in KCl at pH 4 to 4.5 (Cleveland et al., 1983); a trypsin-generated, 187-amino acid C-terminal fragment was tested in NaCl at pH 6 (Bullock et al., 1983). The pore size of channels formed by each of these fragments is also similar to that of the channel formed by whole E1; all of these channels allow passage of ions as large as glucosamine$^+$, glucuronate$^-$, glutathione$^-$, and NAD$^-$ (Raymond, Slatin & Finkelstein 1985*a*). In this paper, we characterize a new 136-amino acid C-terminal fragment of E1 (Liu et al., 1985), designated CNBr(6-1), which forms channels in planar bilayers. The channel formed by this shortest C-terminal fragment also exhibits voltage dependence and ion selectivity (in KCl in pH's around 4) similar to those of the whole E1 channel and has a similar pore size.

Although the characteristics of channels formed by whole E1, ThCF, CNBr, and CNBr(6-1) are almost identical at symmetric low pH in KCl, behavior of the four channels can be distinguished under other conditions. E1 channels have been reported to show pH-dependent gating kinetics (Kagan, 1981; Bullock et al., 1983; Cleveland et al., 1983) and ion selectivity (Raymond et al., 1985*a*). Results presented in this paper show that channels formed by C-terminal fragments of E1 (i.e., ThCF, CNBr, and CNBr(6-1)) exhibit the same trend in pH-dependent gating kinetics and ion selectivity (i.e., turn-on and turn-off rates decrease and selectivity towards cations increases with increasing pH), but that the degree of pH dependence varies with peptide



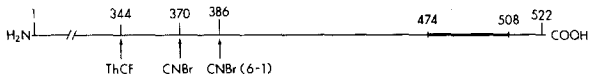


Fig. 1. Schematic diagram of colicin E1, which consists of 522 amino acids. The thermolysin fragment (ThCF) and the two fragments generated by cyanogen bromide cleavage [CNBr and CNBr (6-1)] extend from the cuts marked by their respective arrows to residue 522. (The majority of the thermolysin cuts occur at residue 344, but a small percentage occur at residues 347 and 348.) The 35-amino acid hydrophobic stretch near the C-terminal end (from residue 474 to 508) is indicated in the diagram. *Inset:* Polyacrylamide gel of C-terminal fragments of colicin E1. Lane *1*: CNBr(6-1) fragment; Lane *2*: Wild type CNBr fragment. [In this unpurified preparation, a small amount of the thermolysin fragment (ThCF) from which the CNBr fragment was made is visible.] Lane *3*: Thermolysin fragment (ThCF); Lane *4*: Mol wt markers (from top to bottom: 43 K, 25.7 K, 18.4 K, 13.2 K, 6.2 K, and 3 K)

length. Also, turn-off rates can be distinguished even at symmetric low pH, since high concentrations of the anion glutathione apparently affect the gating kinetics of E1 and ThCF channels more than those of CNBr or CNBr(6-1) channels. (That glutathione affects the turn-off rate of E1 channels has been previously reported by Raymond et al., 1985*a*).

A simple model for E1 channel gating, based on experimental evidence, has been proposed (Cleveland et al., 1983). The model suggests that the 35-amino acid hydrophobic stretch located near the C-terminal end of the E1 molecule spontaneously and irreversibly inserts into the membrane with the C-terminus on the *cis* side, thereby anchoring the protein at the membrane surface. From this configuration, *cis* positive voltage pulses drive the channel-forming portion of the protein into the membrane, to form an open pore. Data consistent with this model were presented in the previous paper (Slatin, Raymond & Finkelstein, 1986). In this paper, we report further evidence to support this model of channel gating, which involves movement of large protein segments into and out of the membrane. Trypsin digestion of membrane-bound channels is shown to convert the behavior of longer peptides to that characteristic of shorter fragments; these and other results to be presented suggest that portions of the E1 molecule not involved in channel formation (i.e., N-terminal to the channel-forming region) are translocated across the membrane during transitions between open and closed states, and that these N-terminal segments modulate gating kinetics and selectivity of the channel. A preliminary report of some of these results appeared earlier (Raymond et al., 1985*b*).

Materials and Methods

Membranes were formed at room temperature from two lipid monolayers of asolectin as described in the previous paper (Slatin et al., 1986). The subsequent addition of colicin E1 or one of its C-terminal fragments and the electrical measurements were also as described there.

The CNBr(6-1) fragment was prepared from a mutant colicin E1 protein, in which a methionine residue was substituted for Val_{386}. This mutant E1 was then cleaved with CNBr to generate the C-terminal fragment containing Asn_{387} through Ile_{522}.

Mutant 386met was made by oligonucleotide-directed site-specific mutagenesis according to the method of Wallace et al. (1980) with the following modification: The oligonucleotide was synthesized with an Applied Biosystem Model 380A DNA synthesizer. Restriction endonuclease SmaI was used to linearize the plasmid DNA, and the double-stranded linear DNA was partially digested with ExoIII to produce an overhanging single-stranded end. The oligonucleotide hybridizes to this single-stranded region, and when treated with Klenow fragment of DNA polymerase I and ligated, a circular plasmid with one mutant strand is obtained with moderate frequency. The oligonucleotide used produces a Met (atg) codon instead of the Val (gtg) codon at amino acid residue 386 of colicin. 386met colicin was prepared from strain JC411 by the method of Cleveland et al. (1983). 10 mg of the purified 386met colicin was digested with thermolysin (colicin: thermolysin weight ratio 100 : 1, at 37°C for 2 hr), and the thermolysin-resistant fragment was purified by gel filtration through a 1.5 cm by 90 cm Sephadex G-50 column. Elution was performed in 25 mM Tris-HCl buffer, pH 7.4, plus 1 mM β-mercaptoethanol. The 18-kD COOH-terminal fragment was then treated with CNBr, and the COOH-terminal CNBr fragment was isolated by preparative SDS polyacrylamide gel electrophoresis as previously described. The size and purity of the thermolysin peptide and CNBr fragment were determined by SDS-polyacrylamide gel electrophoresis with 15% acrylamide and 0.8% bisacrylamide and 8 M urea (*see* Fig. 1 inset). Additional mutants and further details will be described elsewhere (Q.R. Liu et al., *in preparation*).

Trypsin Digestion of Colicin on the Membrane

Type III Trypsin from bovine pancreas and its inhibitor, N-*p*-tosyl-L-lysine chloromethyl ketone (TLCK), both from Sigma Chemical Co., St. Louis, Mo., and Soybean Trypsin Inhibitor, SI 1AA, from Worthington Biochemical, Freehold, N.J., were all stored dessicated at 0°C. Usually a half hour or less before addition to the bilayer, several milligram aliquots of these chemicals were weighed out and then solubilized in buffered KCl solutions (pH 6-7, containing 5 mM $CaCl_2$) to a concentration of 100 mg/ml. In experiments in which inhibitors were used, the trypsin solution was mixed with the appropriate amount of inhibitor solution and incubated at room temperature for at least 5 min before addition to the *cis* or *trans* compartment. Trypsin was mixed

with TLCK in the molar ratio of ca. 1 : 13 (Walter Troll, *personal communication*) and with Soybean Trypsin Inhibitor in the weight ratio of ca. 2.1 : 1 (as suggested on product label). Control experiments, in which TLCK alone was added to one side of a colicin-modified bilayer, showed that the inhibitor itself had no effect on the gating kinetics of colicin.

All experiments began at symmetric low pH (either 3.6 or 4.3) in salt solutions (0.1 to 1 M) buffered with 2–5 mM dimethylglutaric acid (DMG) and also containing 5 mM $CaCl_2$. Prior to the addition of trypsin to one side of the membrane, the pH of that side was raised to around 7, which is within the pH range of maximum trypsin activity (Goldstein, 1976). The final concentration of trypsin used was ca. 1 mg/ml when added to the *cis* compartment, and 2–20 mg/ml when added to the *trans* compartment.

Selectivity Measurements

Membranes were formed in the presence of a 10 : 1 (1 : 0.1 M) gradient of KCl or NaCl, buffered at pH 4.3 with 5 mM DMG and also containing 5 mM $CaCl_2$. Glucose (1.8 molal) was added to the low salt solution in order to avoid streaming potentials, or polarization potentials resulting from dilution of the high salt solution and concentration of the low salt solution at the membrane/solution interfaces due to osmotic water flow across the membrane (Rosenberg & Finkelstein, 1978). Reversal potentials were determined by measuring the voltage necessary to zero the macroscopic current. After the reversal potential at low pH (symmetric 4.3) was determined, the pH was raised symmetrically up to pH 7, either in steps of 0.3 to 1 pH unit or all at once, using concentrated Tris or HEPES buffer solutions.

Reversal potential measurements with 10 : 1 concentration gradients of K^+ glucuronate$^-$, K^+ glutathione$^-$, and K^+ NAD^- (in order to determine pore size of the CNBr(6-1) fragment) were made as described by Raymond et al. (1985*a*).

Results

Quantitative Comparisons of the Properties of Channels Formed by Whole E1 and Its Fragments

Although channels formed by each of the four peptides (E1, ThCF, CNBr, and CNBr(6-1)) are similar in voltage dependence and exhibit similar trends in pH-dependent selectivity and gating kinetics, their behavior can be distinguished under appropriate conditions.

Gating Kinetics as a Function of pH

As noted in the Introduction, turn-on and turn-off rates of channels formed by all four peptides are a function of pH, with turn-off rates influenced mainly by *trans* pH (*unpublished observations*) and turn-on rates by *cis* pH (Bullock et al., 1983; S.L. Slatin, *unpublished results*). The channels formed by E1 show the steepest pH-dependent rates, whereas those formed by CNBr(6-1) show the least.

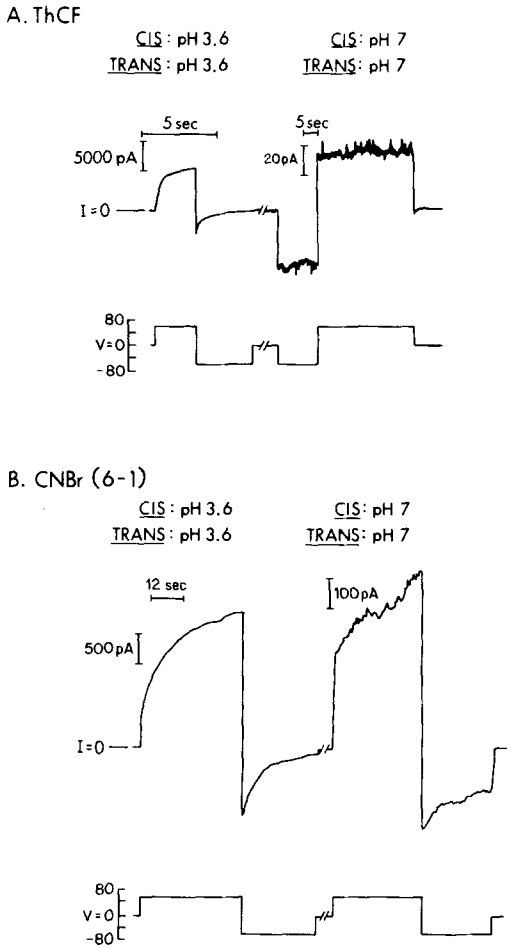


Fig. 2. Effect of pH on turn-on kinetics of ThCF- and CNBr(6-1)-induced conductance. (*A*) *ThCF*, 0.05 μg/ml; (*B*) *CNBr(6-1)*, 0.05 μg/ml. Both records were taken from asolectin membranes which separated symmetric 0.1 M (*A*) or 1 M (*B*) KCl solutions buffered with 2 mM DMG at pH 3.6 and also containing 5 mM $CaCl_2$. Protein was added to the *cis* compartment. The first part of both *A* and *B* (before the break in the records) shows that at symmetric low pH, turn-on and turn-off rates at ±60 mV were rapid (on a time scale of a few seconds or less for turn off at −60 mV). At the break in each record, all channels were turned off, and then the pH was raised symmetrically to 7 (with HEPES, to a concentration of ca. 40 mM in *A*, and with Tris, to a concentration of ca. 5 mM in *B*). (*A*) At symmetric pH 7 (after the break), the only conductance left in the membrane was the voltage-independent leak; ThCF channels no longer turned on at +60 mV (note change in current and time scales after break). (*B*) CNBr(6-1) channels could still be turned on at symmetric pH 7 with a pulse of +60 mV, but the rate of turn on was ca. five times slower than at symmetric pH 3.6 (the initial rapid rise in conductance was due to residual conductance from a previous pulse after the pH change). There was little effect on the turn-off rate. The records were filtered at 10 Hz (*A*) and 2 Hz (*B*)

At around pH 7, both turn-on and turn-off rates decrease monotonically with increasing peptide size; i.e., rates decrease in the order: CNBr(6-1) > CNBr > ThCF > E1. For example, Fig. 2 compares the decrease in turn-on rates (at V = +60 mV) for

Table 1. τ_{off} at *cis* pH 3.6, *trans* pH7[a]

Peptide fragment	Voltage (mV)	Before trypsin			After trypsin		
		#[b]	τ_{off} ± SD (sec)	Extremes	#[b]	τ_{off} ± SD (sec)	Extremes
CNBr(6-1)	60	3	24 ± 2	22;26	1	16	—
	80	3	19 ± 0.6	18;19	2	13 ± 3	11;15
CNBr	60	4	100 ± 19	77;120	3	19 ± 7.5	12;27
	80	4	51 ± 8	40;59	3	14 ± 3.5	10;16
ThCF	60	3	180 ± 30	159;214	3	23 ± 3.5	19;25
	80	3	110 ± 38	68;143	3	16 ± 3	14;19
E1	60[c]	1	240		1	19	—
	80	2	490 ± 69	437;535	1	150	—
	80	2	1400 ± 300	1625;1200	2	110 ± 9	103;116

[a] Unless otherwise noted, the data were obtained in symmetric 1 M KCl.
[b] Denotes number of experiments.
[c] These data were obtained in symmetric 0.1 M KCl.

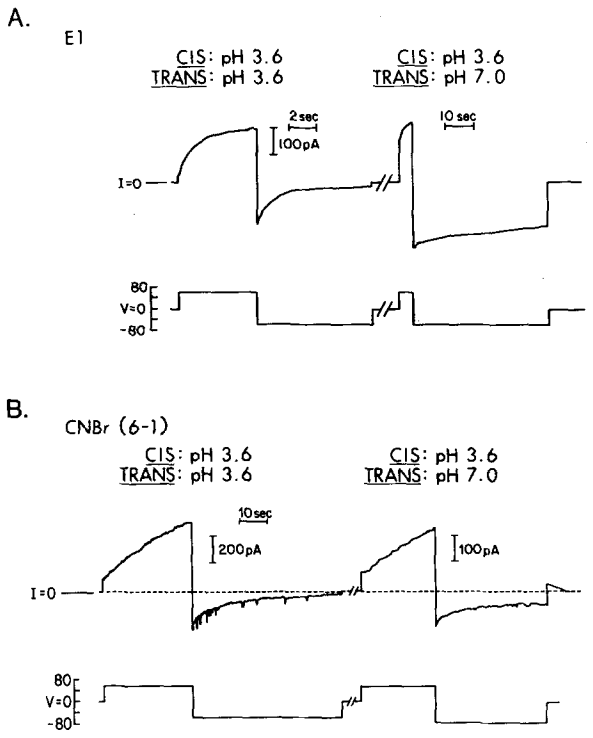


Fig. 3. Effect of *trans* pH on turn-off rates of E1- and CNBr(6-1)-induced conductance. (*A*) E1, 0.04 μg/ml; (*B*) CNBr(6-1), 0.007 μg/ml. An asolectin membrane separated symmetric 0.1 M (*A*) or 1 M (*B*) KCl solutions at pH 3.6, containing 5 mM DMG and 5 mM $CaCl_2$. Protein was added to the *cis* compartment. The representative current responses shown in both *A* and *B* were to pulses of +60 and −60 mV. Before the break in each of these records, turn-off rates are seen to be rapid for both E1 and CNBr(6-1) channels. At the break, the *trans* pH was raised to 7 (with HEPES, to a concentration of ca. 40 mM in *A*, and with Tris, to a concentration of ca. 5 mM in *B*); after this, turn off of E1 channels was dramatically slowed (note change in time scale in *A*), while that of CNBr(6-1) channels was relatively unaffected. The records were filtered at 30 Hz (*A*) and 2 Hz (*B*)

channels formed by ThCF and CNBr(6-1), when the pH was raised symmetrically from 3.6 to 7. Note that no channel activity was detectable (macroscopically) at symmetric pH 7 for ThCF, whereas CNBr(6-1) channels continued to turn on under these conditions, though at a rate about five times slower than that at pH 3.6. Likewise, Fig. 3 compares the effect of *trans* pH on turn-off rates of channels formed by E1 and CNBr(6-1). When the *trans* pH was raised from 3.6 to 7, the turn-off rate at −60 mV was slowed dramatically for E1 channels, but was virtually unchanged for CNBr(6-1) channels. (The quantitative analysis of τ_{off}[1] for the different peptide channels at *trans* pH 7 is described later—*see* Fig. 6 and Table 1.) Experiments such as these with each of the four peptides indicate that τ_{off} at *cis* pH 3.6, *trans* pH 7 increases with peptide length, but the difference in τ_{off} between CNBr(6-1) channels and all the others is the most striking. (The differences in peptide lengths are: 344 amino acids between E1 and ThCF, 26 amino acids between ThCF and CNBr, and 16 amino acids between CNBr and CNBr(6-1).)

In the records shown in Figs. 2 and 3 of ThCF- and CNBr(6-1)-induced conductance at low and high pH, the conductance appears to rectify when the voltage is switched from positive to negative values; the instantaneous conductance at negative voltages appears smaller than that at the corresponding positive voltages. At pH 3.6, this "rectification" reflects the limitations of the chart recorder; when monitored on an oscilloscope, two time constants can be resolved, with the faster one

[1] Turn off does not actually follow a single exponential, but there is one term that dominates (*see* Fig. 6).

Table 2. Reversal potentials (V_r) at pH 7[a] (1 M *vs.* 0.1 M KCl)

Peptide fragment	Before trypsin			After trypsin		
	#	$V_r \pm$ SD (mV)	Extremes (mV)	#	$V_r \pm$ SD (mV)	Extremes (mV)
ThCF	6	41.8 ± 1.9	39.5;45	6	26.1 ± 0.7	25.5;27.5
CNBr(6-1)	2	27 ± 3.5	24.5;29.5	1	26	—

[a] Reversal potentials measured in a 10:1 concentration gradient were normalized to a 10:1 activity gradient. The potential of the 0.1 M KCl solution is positive with respect to the 1 M KCl solution for all entries.
# Denotes number of experiments.

on the order of a few milliseconds (Slatin & Finkelstein, 1984). Turn off at pH 7 was not analyzed at a time resolution higher than 100 Hz, but this kind of rectification has been reported to occur for channels formed by both whole E1 and its trypsin fragment on a time scale of less than 1 msec at pH 6 (Bullock et al., 1983). The CNBr(6-1) conductance, which appears to turn off rapidly at pH 7, also shows an initial "on" conductance that is two to three times the "off" conductance seen just prior to the voltage switch. We have not investigated whether this rectification is explained by fast components of turn-on and turn-off kinetics, or if it occurs at the level of single-channel conductance.

Selectivity as a Function of pH

Titration curves of selectivity *vs.* pH for each of the four peptides were obtained over a pH range from 4.3 to 7 (*data not shown*). As with gating kinetics, the selectivity of channels formed by E1 shows the steepest pH dependence, whereas the selectivity *vs.* pH curves for the channels formed by the two cyanogen bromide fragments are the most shallow. Channels formed by all four peptides are anion preferring at low pH, with a ratio of P_{Cl}/P_K of about 4:1 at pH 4.3. At higher pH's, however, the difference in reversal potentials is notable (*see* Table 2); although all the channels are cation-preferring at pH 7, the reversal potential of E1 channels for 1 *vs.* 0.1 M KCl at pH 7 (49.3 ± 0.4 mV) is nearly 10 mV higher than that of ThCF channels (41.8 ± 1.9 mV), which in turn is about 10 to 15 mV higher than that of channels formed by the cyanogen bromide fragments (32 mV for CNBr channels and 27 ± 3.5 mV for CNBr (6-1) channels).

Effect of Glutathione on Gating Kinetics

It had previously been noted that glutathione has a "pharmacological" effect on E1 channels (Raymond et al., 1985*a*). Experiments were now conducted to determine the effect of glutathione concentration on gating kinetics of channels formed by E1 and its fragments under four different salt conditions, all at symmetric pH 4.3: (i) symmetric 0.1 M K-glutathione, (ii) symmetric 1 M K-glutathione, (iii) *cis* 1 M K-glutathione, *trans* 1 M KCl, and (iv) *cis* 1 M KCl, *trans* 1 M K-glutathione. Solutions were buffered with 2–5 mM DMG and contained 5 mM $CaCl_2$, as in other experiments.

Turn-on rates of E1 channels were unaffected under any of the four conditions. Turn-off rates, however, were slowed dramatically under conditions ii and iv (from a τ_{off} in symmetric KCl, pH 4.3 of 30 sec to a τ_{off} of around 210 sec, at −60 mV—*see* Fig. 4*A* and *B*, respectively), but were unaffected under conditions i and iii. Thus, high concentrations of glutathione (ca. 1 M) on the *trans* side significantly slow the voltage-dependent τ_{off} of channels formed by E1; *cis* glutathione is without effect on channel gating. E1 channels can be turned off completely in less than 1 min in the presence of *trans* 1 M K-glutathione, however, with voltage pulses of about −150 mV (*not shown*). Figure 4 compares turn-off rates for E1 and ThCF channels in *cis* 1 M KCl, *trans* 1 M K-glutathione with those seen in symmetric 1 M KCl. As already noted, τ_{off} for E1 channels is slowed 5- to 10-fold in *trans* 1 M K-glutathione; in contrast, τ_{off} for ThCF channels is slowed only by a factor of 2 to 3 (it is primarily the fast phase of turn off which is affected). Finally, turn-off rates of channels formed by the cyanogen bromide fragments are unaffected by *trans* 1 M K-glutathione (*data not shown*).

Pore Size

We previously found that the channels formed by whole colicin E1, ThCF, and CNBr all have comparable lumen sizes, based on the observation that reversal potentials (V_r) in monovalent salts of a

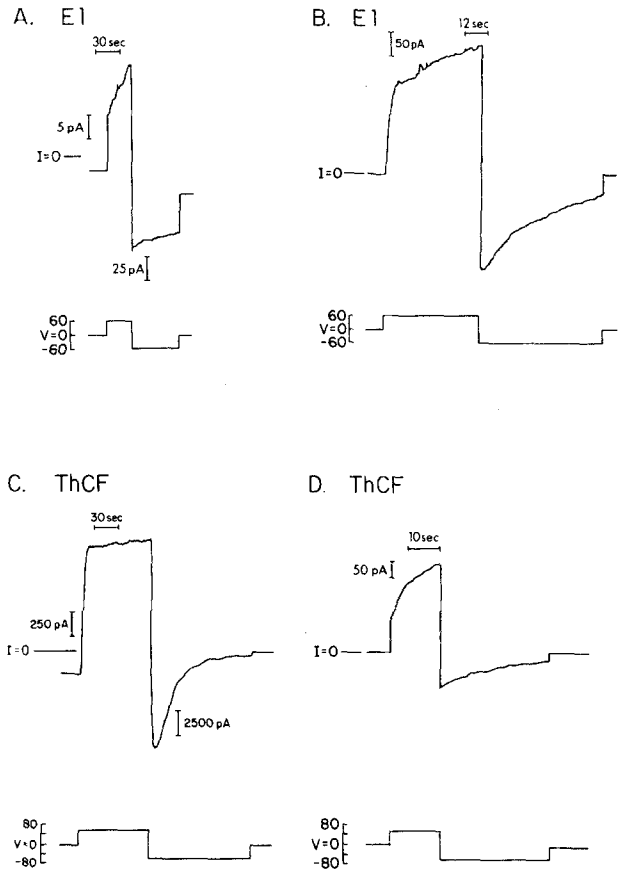


Fig. 4. Kinetics of turn off in *cis* 1 M KCl, *trans* 1 M K-glutathione *vs.* turn off in symmetric 1 M KCl for E1 and ThCF channels. An asolectin membrane separated salt solutions buffered at pH 4.3 with 2 mM DMG and containing 5 mM $MgSO_4$. Protein was added to the *cis* side. The current traces shown in *A–D* are representative responses to pulses of +60 and −60 mV. (*A*) E1, 0.40 μg/ml, in *cis* 1 M KCl, *trans* 1 M K-glutathione. (*B*) E1, 0.05 μg/ml, in symmetric 1 M KCl. In both *A* and *B*, some channels had been turned on prior to the portion of the record shown, but these traces show that turn on of additional E1 channels occurred at comparable rates for these two different salt conditions. Turn off, however, was much slower in the trace shown in *A* than in that shown in *B*. (Note the difference in time scales between *A* and *B*. Also, the initial current at −60 is much greater than that at +60 mV in trace *A* because the current is proportional to conductance times the driving force, $V - V_r$; at low pH, the E1 channel prefers anions, and Cl^- is significantly more permeant than is glutathione, resulting in a non-zero bi-ionic potential (V_r)). (*C*) ThCF, 0.35 μg/ml, in *cis* 1 M KCl, *trans* 1 M K-glutathione. (*D*) ThCF, 0.05 μg/ml, in symmetric 1 M KCl. Again, channels had been turned on before the beginning of the traces shown in *C* and *D* (in *C*, conductance had reached steady state). Turn-off rates are seen to be somewhat slower for ThCF channels in *trans* 1 M K-glutathione (*C*) than in *trans* 1 M KCl (*D*). (Again, note the difference in time scales between *C* and *D*, and as in *A*, the asymmetry of current at +60 and −60 mV seen in *C* is explained by the non-zero bi-ionic potential)

large and a small ion are less than the Nernst potential for the small ion (Raymond et al., 1985*b*). For example, the reversal potential for channels formed by whole colicin E1 in a 10 : 1 K^+ glucuronate$^-$ gradient at pH 4.4 was only +38 mV, whereas the Nernst potential for K^+ was +52.5 mV. The values given in Table 3 were obtained for the reversal potentials of the CNBr(6-1) channel in 10 : 1 gradients of three different salts. Evidently, the CNBr(6-1) channel, like the channels formed by whole E1, ThCF, and CNBr, are permeable to glucuronate$^-$, glutathione$^-$, and NAD^-.

Table 3. Reversal potentials for the CNBr(6-1) channel

pH	Salt	V_r (mV)	Nernst potential (mV) for K^+
4.4	K^+ glucuronate$^-$	39.5	52.5
4.3	K^+ glutathione$^-$	47	54
4.2	K^+ NAD^-	44	53

Trypsin Conversion of Longer Peptides to Shorter Peptides

Given that basic channel properties (voltage dependence, pore size, and ion selectivity) are qualitatively the same for channels formed by each of the four peptides, the quantitative differences in behavior documented above imply that amino acids N-terminal to the channel-forming region influence the pH dependence of gating kinetics and selectivity. Furthermore, the fact that glutathione slows turn-off rates preferentially (i.e., it has the most significant effect on E1 channels and little or no effect on CNBr and CNBr(6-1) channels), and only from the *trans* side, suggests that some of these N-terminal amino acids are on the *trans* side of the membrane, at least when the channel is in the open state. The following experiments, in which trypsin was added to either the *cis* or the *trans* bathing solution of a colicin-modified bilayer, support this hypothesis.

Trypsin Digestion from the Trans Side

To compare the turn-off rates of channels formed by E1, ThCF, CNBr, and CNBr(6-1) at *trans* pH 7 (*cis* pH 3.6) before and after trypsin digestion from the *trans* side, experiments such as those illustrated in Fig. 5*A* and *B* (with CNBr(6-1) and ThCF, respectively) were carried out. At *cis* pH 3.6, *trans* pH 7 (the beginning of the records shown in Fig. 5), the turn-off rate at −60 mV was still rapid for CNBr(6-1) channels (Fig. 5*A*) but was slowed considerably for ThCF channels (Fig. 5*B*), as compared to rates at symmetric pH 3.6. Less than 1 min after trypsin addition to the *trans* side, however, the turn-off rate of ThCF channels speeded up significantly, as evidenced by the representative pulses to ±60 mV shown in Fig. 5*B*. When the *cis* pH was

raised to 7 many minutes later (at the second break in the record shown in Fig. 5*B*), ThCF channels still turned on with *cis* positive voltage, behavior characteristics of channels formed by the CNBr(6-1) fragment (*see* Fig. 2)[2]. Addition of trypsin to the *trans* side of the CNBr(6-1)-modified membrane, on the other hand, had little effect on turn-off kinetics (Fig. 5*A*). Control experiments in which a mixture of trypsin and an inhibitor (either TLCK or Soybean Trypsin Inhibitor) were added to the *trans* side showed that both inhibitors prevented the trypsin-induced increase in turn-off rates; in both cases, when an excess of trypsin was subsequently added (to the same compartment), it effected a significant decrease in τ_{off}.

Turn-off rates for each of the four peptides at *trans* pH 7, before and after *trans* trypsin treatment, were analyzed by plotting ln $(g(t)/g_i)$ *vs.* time at −80 mV (where g_i is the initial conductance just before the voltage was switched from *cis* positive to *cis* negative values, and $g(t)$ is the conductance at time t after the voltage switch). These plots are shown in Fig. 6; each line represents data from one membrane, and average τ_{off}'s were determined from the slopes. Table 1 compares the time constants for each of the four peptides. The data shown in Fig. 6 were obtained from experiments carried out in 1 M KCl at *cis* pH 3.6, *trans* pH 7; trypsin was added to the *trans* side at a concentration of 10 to 20 mg/ml. It is clear from these plots and from Table 1 that before addition of trypsin, both CNBr and ThCF channels turn off at much slower rates than CNBr(6-1) channels, but that within 5 to 10 min after addition of trypsin to the *trans* side, CNBr and ThCF channels show τ_{off}'s comparable to those of CNBr(6-1) channels. The turn-off rate of E1 channels, however, does not increase to that characteristic of CNBr(6-1) channels even after incubation for up to a half hour with trypsin on the *trans* side (*see* Fig. 6*D*). The inability to reach the same end-state with E1 channels as that attained with ThCF channels was troublesome, and neither increasing the trypsin concentration nor the incubation time significantly improved trypsin's effectiveness (from the *trans* side) in converting E1 channels to the behavior of CNBr(6-1) channels. In symmetric 0.1 M instead of 1 M KCl, however, lower concentrations of trypsin (ca. 2 mg/ml) were effective in converting ThCF channels to CNBr(6-1)-like channels, and were also effective in making this conversion of E1 channels in ca. 5 min (*see* Fig. 7 and Table 1). There is no reason to believe that trypsin activity in this system is inhibited at high (1 M) salt concentrations; rather, the differences in trypsin action at high and

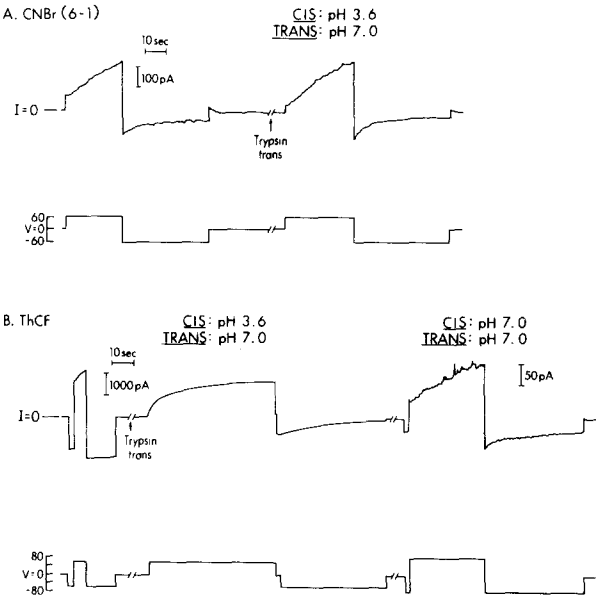


Fig. 5. Effect of trypsin added to the *trans* side on the turn-off rates at *trans* pH 7 of CNBr(6-1)- and ThCF-induced conductance. An asolectin membrane separated symmetric 1 M (*A*) or 0.1 M (*B*) KCl solutions at pH 3.6, containing 5 mM DMG and 5 mM $CaCl_2$. Protein was added to the *cis* side, conductance was established, and then the *trans* pH was raised to 7 (with Tris buffer, to a concentration of ca. 5 mM). (*A*) CNBr(6-1) at a concentration of 0.007 μg/ml: At *cis* pH 3.6, *trans* pH 7, the greater proportion of conductance that was turned on at +60 mV is seen to turn off at −60 mV on a time scale too rapid to be resolved (filtering at 10 Hz, but resolution of this turn off is not much better at 100 Hz). At the break in the record, trypsin was added to the *trans* side to a concentration of 15 mg/ml. Some minutes after trypsin addition, conductance was turned on and off at ±60 mV, and both turn-on and turn-off rates appear to be unchanged. (*B*) ThCF at a concentration of 0.04 μg/ml: After the *trans* pH was raised to 7, conductance could be seen to turn on with a pulse of +60 mV, but very little of this conductance turned off in 15 sec at −60 mV. At the first break in the record, trypsin was added to the *trans* side to a concentration of 2 mg/ml, and a few minutes later, conductance that was turned on at +60 mV turned off rapidly at −60 mV. Several minutes later, the pH of the *cis* side was increased to 7 (at the second break). At symmetric pH 7, the conductance still turned on (and off) at a reasonable rate—comparable to that of CNBr(6-1) channels. (The much lower activity at *cis* pH 7 is also seen for CNBr(6-1) channels—*see* Fig. 2*B*). This figure shows that the turn-off rate of ThCF channels is much slower than that of CNBr(6-1) channels at *cis* pH 3.6, *trans* pH 7, and that trypsin added to the *trans* side under these conditions speeds up the turn-off rate of ThCF channels but has little effect on the turn-off rate of CNBr(6-1) channels

[2] When pepsin is added to the *trans* side of a ThCF-treated membrane at symmetric low pH (3.5), there is no discernible effect on channel behavior (Slatin et al., 1986). If, however, the trans pH is subsequently raised to pH 7, some of the channels (unlike normal ThCF channels) turn off rapidly as if they have been converted to CNBr(6-1) channels. In addition, if the *cis* pH is subsequently raised to 7, the turned off channels can be turned on by positive voltages, again a behavior characteristic of CNBr(6-1) channels rather than of ThCF channels. Thus, the actions of pepsin and trypsin from the *trans* side are similar, although we have never been able to convert all of the ThCF channels in the membrane to CNBr(6-1)-type channels with pepsin.

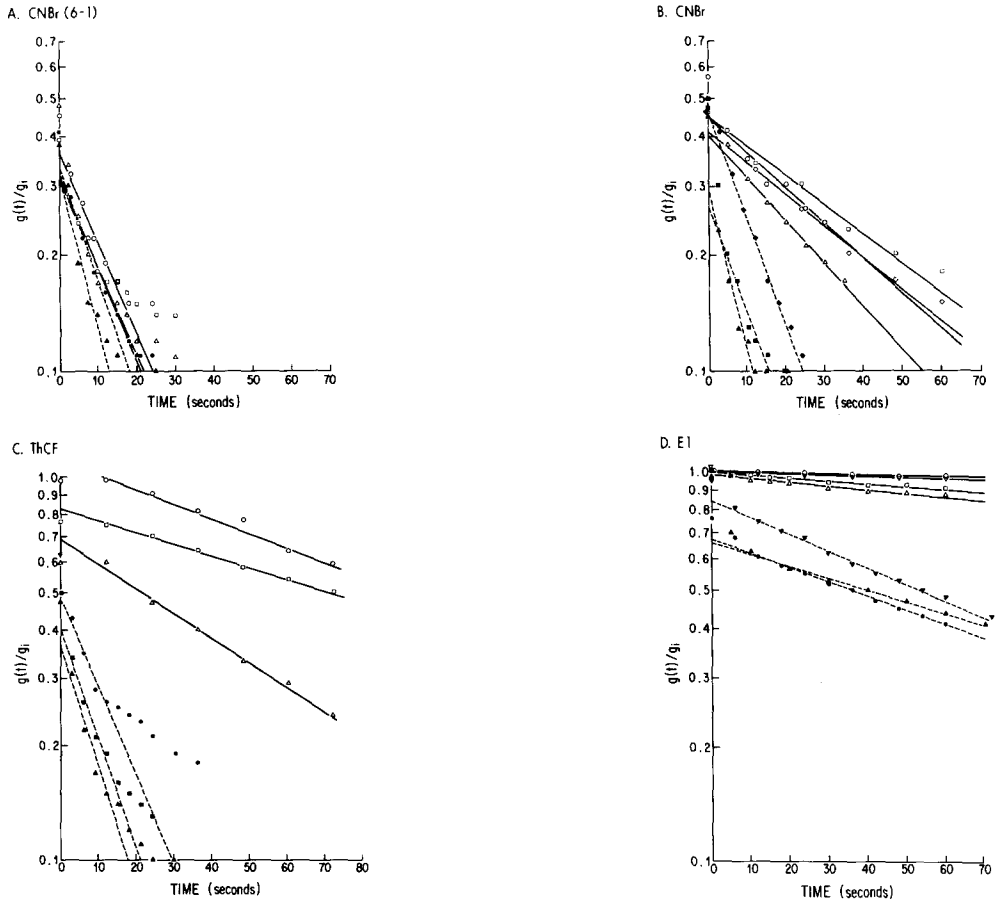


Fig. 6. Turn-off rates at *cis* pH 3.6, *trans* pH 7 before and after trypsin addition to *trans* side, $g(t)/g_i$ is plotted logarithmically on the ordinate, where $g(t)$ is the conductance at time t after switching the voltage polarity from +80 to −80 mV, and g_i is the membrane conductance at the time of the voltage change. Open symbols connected by solid lines represent turn off before trypsin addition, and closed symbols connected by dashed lines represent turn off after trypsin addition. (*A*) CNBr(6-1); (*B*) CNBr; (*C*) ThCF; (*D*) E1. All experiments were carried out on asolectin membranes, to which 0.01 to 0.2 μg/ml protein was added. The salt solutions were symmetric 1 M KCl; these solutions were buffered at pH 3.6 with 2–5 mM DMG and also contained 5 mM $CaCl_2$. Experiments began at symmetric pH 3.6, and after channels had been turned on, the *trans* pH was raised to 7 with a concentrated HEPES solution (to a final concentration of ca. 40 mM; in some cases, Tris was used instead, at a concentration of ca. 5 mM). After the turn-off rate was established, trypsin was added to the *trans* side (to a final concentration of 10 to 20 mg/ml). Channels were pulsed on and off over a 5 to 10 min period, and turn-off rates were then measured for a single pulse. Each curve (i.e., different symbol) represents a single membrane, and the $g(t)/g_i$ points were obtained from a single turn-off pulse. These curves illustrate that turn-off rates at a given voltage under these conditions decrease with increasing peptide length, in the order: CNBr(6-1) > CNBr > ThCF > E1, and that the turn-off rates of the latter three are increased following the action of trypsin from the *trans* side. The turn-off rate is not a single exponential; for E1 and ThCF after trypsin and for CNBr and CNBr(6-1) both before and after trypsin, there is a very fast rate through the first two or three seconds, followed by an intermediate rate (which is what the lines represent), and finally, on some membranes, a much slower rate for the last 10 to 30% of the conductance. The fastest component of turn off cannot be resolved with 100 Hz filtering, and this is reflected in the low ratio of $g(t)/g_i$ at $t = 0$

low salt concentrations probably reflect the effect of salt concentration on the conformation of the extra-channel domain exposed on the *trans* side.

Figure 8*A* represents a variation on the experiments shown in Fig. 5*A* and *B*. ThCF channels were held in the closed state during a 2 to 3 min incubation with *trans* trypsin, and then TLCK was stirred into the *trans* compartment before channels were pulsed open. This protocol restricted the substrate available for *trans* trypsin digestion to channels in the closed state. The turn-off rate of ThCF channels was unchanged after the trypsin incubation (*compare* Fig. 8*A* to 5*B* before addition of trypsin), indicating that *trans* trypsin is unable to modify channels that are in the closed state. As a control, channels were pulsed to ±60 mV after being held

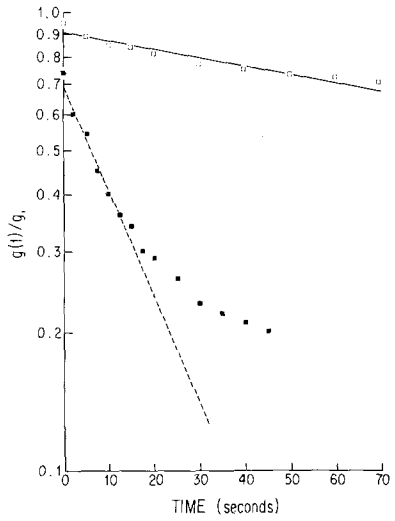


Fig. 7. Turn-off rate of E1 channels in 0.1 M KCl at *cis* pH 3.6, *trans* pH 7 before and after trypsin addition to *trans* side. $g(t)/g_i$ *vs.* time is plotted semilogarithmically as described in Fig. 6, except that the voltage pulse used was −60 mV. The two lines represent data obtained from one membrane, the broken line representing turn off about 5 min after addition of 2 mg/ml trypsin to the *trans* side. E1 was added to the *cis* side of an asolectin membrane to a concentration of 0.03 μg/ml; the salt solutions were symmetric 0.1 M KCl, 5 mM DMG, 5 mM $CaCl_2$, pH 3.6. The protocol of this experiment was as described in Fig. 6. This figure shows that the turn-off rate for E1 channels in symmetric 0.1 M KCl at *trans* pH 7 speeds up significantly after *trans* trypsin addition; in fact, τ_{off} after trypsin is comparable to that seen for CNBr(6-1) channels at *trans* pH 7 (*see* Table 1). Note that the turn-off rate before trypsin addition seen here is faster than that seen for E1 channels in 1 M KCl under identical pH conditions (*compare* with Fig. 6*D*)

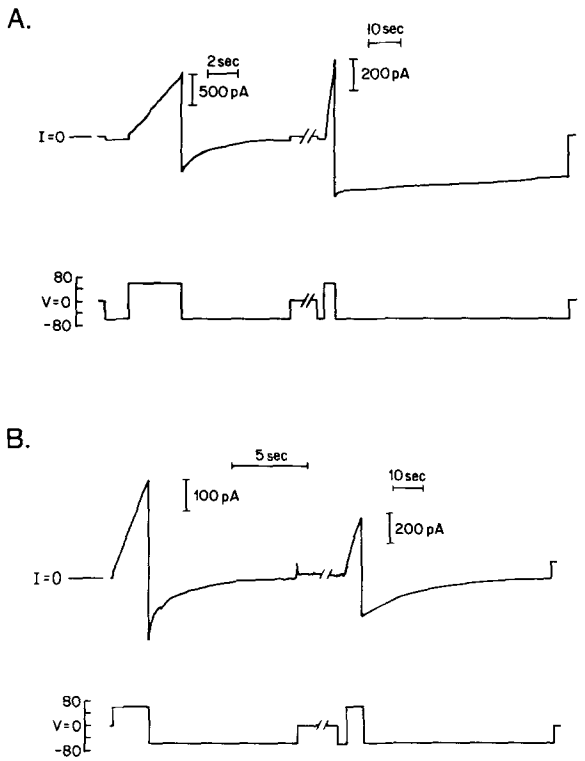


Fig. 8. Ineffectiveness of *trans* trypsin on closed ThCF channels. An asolectin membrane separated symmetric 0.1 M KCl solutions containing 5 mM DMG and 5 mM $CaCl_2$ at pH 3.6. ThCF was added to the *cis* side to a concentration of 0.05 μg/ml (*A*) or 0.025 μg/ml (*B*), and channel activity was established at symmetric pH 3.6 (pulses are ±60 mV). (*A*) Conductance was turned off to baseline and then held off with a pulse of −30 mV all during the break in the record. During this time, the *trans* pH was raised to 7 (with HEPES, to a final concentration of ca. 40 mM), trypsin was added to the *trans* side to a concentration of 2 mg/ml, and 3 min later, TLCK was added to the same side to a concentration of 0.7 mg/ml. Three minutes after addition of TLCK, channels were turned on with a +60-mV pulse (just after the break in the record). A pulse of −60 mV did not turn off this conductance rapidly; instead, conductance is seen to turn off at a rate typical of unmodified ThCF channels (*compare* with Fig. 5*B*, before the addition of trypsin). (*B*) As in *A*, the conductance was held off at −30 mV all during the break, while the *trans* pH was raised to 7 (with Tris to a final concentration of ca. 5 mM) and while trypsin was added to the *trans* side to a concentration of 2 mg/ml. Two minutes after trypsin addition, some conductance was turned on with a pulse of +60 mV for 5 sec, and then turned off at −60 mV over a 1-min period, showing that trypsin had "chewed" ThCF channels in the time it took to turn the conductance on and off. Two points are illustrated in this figure: (1) trypsin does not act on closed ThCF channels from the *trans* side over an incubation period of 3 min, and (2) trypsin modifies open channels from the *trans* side within seconds

closed during the 2 to 3 min incubation with *trans* trypsin (*see* Fig. 8*B*). The rapid turn off seen in this experiment contrasts with the slow turn off, characteristic of unmodified ThCF channels, seen in the previous one (Fig. 8*A*). From these experiments, it is clear that although *trans* trypsin works in seconds to convert *open* ThCF channels to CNBr(6-1)-like channels (Fig. 8*B*), it has no such effect on *closed* ThCF channels during a 2 to 3 min incubation period (Fig. 8*A*).

Trypsin Digestion from the Cis Side

Experiments to monitor trypsin digestion of E1 and ThCF channels from the *cis* side of the bilayer were carried out in *cis* 1 M KCl, *trans* 1 M glutathione, starting at symmetric pH 4.3. (As previously described, under these conditions turn-off rates at −60 mV are slowed considerably for E1 channels, whereas those of ThCF channels are only slightly slowed, and turn-off rates of CNBr and CNBr(6-1) are unaffected, as compared with rates seen in symmetric 1 M KCl.) A representative experiment is shown in Fig. 9. Prior to the start of this trace, some E1 channels were turned on; subsequently, the *cis* pH was raised to 7 and trypsin was added to the *cis*

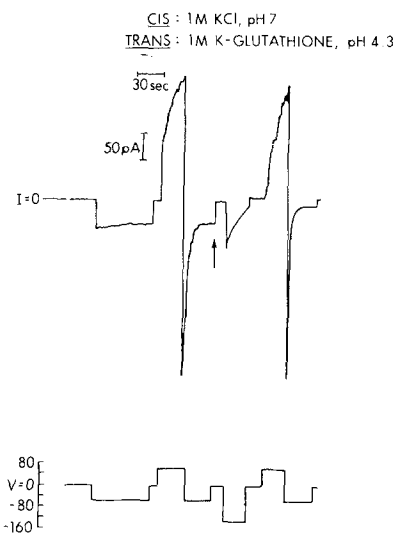


Fig. 9. Effect of trypsin added to the *cis* side on the turn-off rate in *trans* 1 M K-glutathione of E1-induced conductance. E1 was added to the *cis* side of an asolectin membrane to a concentration of 1 μg/ml. Solutions were *cis* 1 M KCl, *trans* 1 M K-glutathione, both containing 2 mM DMG and 5 mM $CaCl_2$, at pH 4.3. Some conductance was turned on, the *cis* pH raised to 7 (with Tris, to a concentration of 5 mM), and trypsin added to the *cis* compartment to a concentration of 0.5 mg/ml. The record shown here begins approximately 15 min after trypsin addition, and the turn off at −60 mV is seen to be very slow. (No new conductance was turned on during the 15-min period.) However, when new conductance was subsequently turned on with a pulse of +60 mV, this conductance turned off rapidly at −60 mV, leaving the "old" conductance still on (noted at arrow). This "old" conductance was then turned off with a pulse of −150 mV; after this, all the conductance is seen to turn on and off rapidly. This figure shows that E1 channels can be modified to CNBr(6-1)-like channels by digestion with trypsin on the *cis* side, but that the protease acts only on closed channels

compartment. No new channels were turned on during a 15-min incubation period (at 0 voltage) with *cis* trypsin, and during this time the turn-off rate of those channels turned on before trypsin addition remained slow (*compare* the first pulse in Fig. 9 (−60 mV) with the turn-off rate shown in Fig 4*A*). When positive voltage (+60 mV) was applied at the end of this 15-min incubation, however, the newly opened channels turned off rapidly at −60 mV. Conductance did not go to zero, however, since the channels that had been turned on before *cis* trypsin addition were still open (the arrow points to this residual conductance). These "unmodified" channels were turned off with a pulse of −150 mV, and thereafter there was only one population of channels in the membrane—all the conductance could be turned on and off rapidly with voltage pulses of ±60 mV. These results indicate that after *cis* trypsin digestion, E1 is converted to a new peptide which forms channels that behave like those of CNBr and CNBr(6-1) (i.e., that turn on at *cis* pH 7 and turn off rapidly in *trans* 1 M glutathione). Moreover, this conversion only occurs when the channels are in the closed state. Similar results were obtained in experiments carried out with ThCF. Control experiments showed that soybean trypsin inhibitor was effective in preventing the trypsin-mediated conversion.

Selectivity

Experiments to determine the ion selectivity at pH 7 of channels formed by ThCF and CNBr(6-1) before and after *trans* trypsin digestion were performed according to the following protocol. Protein was added to the low salt side, and after channels were turned on to achieve a conductance on the order of 10^3 to 10^4 pS, the zero current potential at symmetric pH 4.3 was measured, before the pH was raised on both sides to 7. The zero current potential at symmetric pH 7 was determined, and the membrane was then clamped at this voltage while trypsin was stirred into the *trans* compartment (in order to hold a single population of channels open while being "chewed" by trypsin). Once the deviation of current from the I = 0 line had stabilized (this took 2 to 3 min), the applied voltage was adjusted in order to zero the current at the new reversal potential. It was clear that the decrease in zero current potential resulted from trypsin-mediated conversion of ThCF channels and not from the development of leaks in the membrane, since the membrane conductance could be turned off rapidly with *cis* negative voltages after trypsin addition.

Reversal potentials of channels formed by ThCF and CNBr(6-1), measured at pH 7 as described above, are shown in Table 2. The first column lists reversal potentials measured before the addition of trypsin to the *trans* side of the membrane, and the second column lists the "after trypsin" reversal potentials. The reversal potential of the CNBr(6-1) channel did not change significantly after *trans* trypsin addition, whereas that of the ThCF channel (42 mV) decreased by about 15 mV, making it comparable to the reversal potential of the CNBr(6-1) channel (ca. 27 mV).

Discussion

The results presented in this paper demonstrate that amino acids N-terminal to the channel-forming region of the colicin E1 molecule modulate gating kinetics and ion selectivity of the channel. Since E1 and its C-terminal fragments, ThCF, CNBr and CNBr(6-1), form channels (at low pH) that are qual-

itatively similar in voltage dependence, pore size, and trend in pH-dependent ion selectivity, we assume that the channels formed by these four peptides are also similar, if not identical, in structure. The quantitative differences in pH dependence of gating kinetics and selectivity described in the Results must therefore be attributed to effects mediated by amino acids not involved in channel formation (i.e., those upstream from the C-terminal 136 amino acids). Experiments demonstrating that proteolytic digestion from the *trans* side converts the behavior of E1, ThCF, and CNBr channels to that characteristic of CNBr(6-1) channels, and that this proteolytic digestion occurs when the channels are open but not when they are closed, suggest that these amino acids are translocated across the membrane in conjunction with channel gating.

Aside from differences in pH-dependent gating kinetics and selectivity, channels formed by these four peptides also exhibit differences in voltage-dependent turn-off rates at low pH in the presence of *trans* glutathione. The fact that E1 channels are most affected under this condition, whereas channels formed by CNBr and CNBr(6-1) are least affected, implies that amino acids N-terminal to Ala_{371} (the N-terminus of CNBr) lie on the *trans* side of the membrane when the channel is in the open state. This inference is supported by the "sidedness" of trypsin action on E1 and ThCF channels; evidently, these channels cannot be modified by trypsin from the *cis* side when they are in the open state (Fig. 9), and conversely, cannot be modified by trypsin from the *trans* side when in the closed state (Fig. 8*A*). This suggests that these N-terminal amino acids are on the *cis* side when the channel is closed and that at least some of them are translocated across the membrane with gating. Both the closed state and (two versions of) the open state of the channel are shown schematically in Fig. 10*A*.

There is no *a priori* reason why these N-terminal amino acids should be flipped from one side of the membrane to the other during transitions between open and closed states of the channel, since their movement is not essential to the gating process. They may, however, be translocated as a consequence of the orientation of the channel in the membrane, as indicated in Fig. 10*A*. In other words, the voltage-dependent step in channel opening is insertion of major portions of the channel-forming region of the protein into the membrane; i.e., most of the E1 molecule lies parallel to the membrane surface in the closed state, and part of it is driven across the membrane with positive voltage pulses to form a membrane-spanning pore (as proposed by Cleveland et al., 1983). If the N-terminus of E1's channel-forming segment were to end up on the

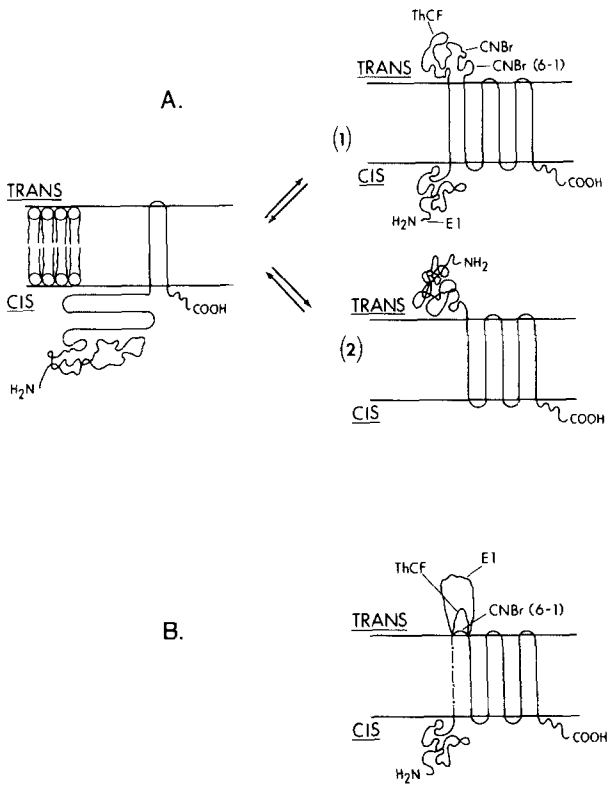


Fig. 10. A model of channel gating for E1 and its C-terminal fragments: Possible orientations of the N-terminal amino acids in the channel's open state. [The location of the C-terminus on the *cis* side is made on the basis of carboxypeptidase Y's ability to affect channel activity from the *cis* side, and not from the *trans* side (Cleveland et al., 1983). Possible endopeptidase activity in the carboxypeptidase preparation introduces some uncertainly in the placement of the C-terminus on the *cis* side.] (*A*) E1 channel gating. In the closed state, the stretch of 35 amino acids near the C-terminus anchors the rest of the molecule at the membrane surface in the absence of voltage. *Cis* positive voltages cause the rest of the channel-forming region of E1 to insert into the membrane to form an open pore, dragging amino acids N-terminal to this region across the membrane as well. Two possible open states are depicted, (*1*) and (*2*), and the N-terminus of each of the four different channel-forming peptides is denoted. (*B*) An alternative view of the open channel. The closed state of the channel is the same as in *A*. The membrane-spanning domain denoted by the broken line represents the "variable" region of the channel structure. The number of amino acids N-terminal to the channel-forming region that are present on the *trans* side of the membrane varies with the identity of the channel-forming peptide (this number is shown here to decrease with decreasing peptide length), and therefore the character of the most N-terminal region of the channel structure is different for the different peptides. This figure is not meant to accurately represent the number of membrane-spanning segments in the open state, except that the number must be odd for the states depicted in *A*, and even for the state depicted in *B*. Domains are unspecified with respect to secondary structure. Also, the membrane-spanning conformation of the E1 molecule represented in these diagrams is not necessarily an open state; that is, there may be some closed states in which the channel spans the membrane and in which N-terminal amino acids are on the *trans* side

trans side after insertion into the membrane, then any amino acids attached to it would be pulled across the membrane as well.

One can estimate how much of the N-terminal portion of E1 is translocated as a consequence of gating, based on differences in behavior between ThCF and E1 channels. It is clear that *trans* 1 M glutathione has a more significant effect on the turn-off kinetics of E1 channels than of ThCF channels, suggesting that some site(s) unique to E1 (i.e., N-terminal to Ile_{345}—*see* Fig. 1) are interacting with glutathione on the *trans* side. Further evidence indicating that there is more protein on the *trans* side for E1 than for ThCF channels in the open state is provided by *trans* trypsin digestion experiments in 1 M KCl; modification of E1 channels is much slower than that of ThCF channels and, in fact, never reaches completion.[3] Taken together, these results suggest that more than 40 amino acids (the difference between ThCF and CNBr(6-1) fragments) are translocated across the membrane during transitions between open and closed states of the channel.

The results of the trypsin digestion experiments, which suggest that a rather large segment of the colicin E1 protein is translocated across the membrane as a consequence of channel gating, are somewhat surprising. Large energy barriers must be overcome in the process of pushing charged and polar amino acids into and across the hydrophobic bilayer. At room temperature these energies are on the order of ten to twenty times kT (the thermal energy) per charged residue transferred[4] from an aqueous environment to that of the hydrophobic lipid bilayer, and values for polar residues are at least several times kT (Engleman and Steitz, 1981). For a hydrophilic molecule like E1, the activation energy costs could be enormous; of the 42 amino acids located between Ile_{345} and Asn_{387} (the N-terminus of ThCF and CNBr(6-1), respectively), there are eight positively charged, six negatively charged, and eleven relatively polar residues. However, four of the basic residues (lysines) are clustered in a six-residue stretch located just five amino acids upstream from the N-terminus of CNBr(6-1); it may be that the *cis* positive voltages which turn on E1 channels facilitate translocation of this N-terminal segment, since these four lysines would be favored by the voltage to cross the membrane.

Precedence for the membrane potential aiding insertion of membrane proteins exists for the M13 procoat protein (Date, Goodman & Wickner 1980) and in the post-translational insertion of protein into the mitochondrial membrane (Hay, Bohni & Gasser, 1984). In the case of M13 procoat protein, which is produced inside host bacteria by coliphage M13, it has been shown that, in the absence of a potential across the bacteria's inner membrane, this precursor protein does not insert into the membrane and undergo processing to form coat protein (Date et al., 1980). The voltage-dependent insertion and subsequent orientation of this molecule in the bacterial inner membrane is thought to be governed by its asymmetric charge distribution (Weinstein et al., 1982). Procoat has a 23-amino acid leader sequence, and this sequence is followed by three negatively charged residues in positions two, four, and five of the mature protein. Therefore, insertion of a helical hairpin which includes the leader sequence and the first 20 or so amino acids of the protein would be favored by the polarity of the potential across the bacterial inner membrane, which is inside negative. There is also one report of voltage-dependent translocation of an entire protein (asialoglycoprotein receptor) across planar lipid bilayer membranes (Blumenthal, Klausner & Weinstein, 1980), and the results in the preceding paper (Slatin et al., 1986) indicate that the entire channel-forming domain of colicin E1 can be translocated across the membrane.

An alternative mechanism for translocation of the "extra" amino acids of E1 is for the segment to thread its way through the channel lumen. That is, the sequence of residues N-terminal to the channel-forming segment may be enclosed within the space that will become the lumen, attached to a point which ends up on the *trans* side when the channel-forming segment flips into the membrane; the enclosed amino acids would then move sequentially through the lumen to emerge on the *trans* side of the membrane in order to "unplug" the pore. This model is reminiscent of the water-filled pore mechanism proposed for co-translational translocation of proteins through the ER membrane (Blobel & Dobberstein, 1975). This alternative for E1 is less likely

[3] As indicated in the Results, trypsin activity in this system is apparently diminished, although it is unclear whether this effect seen in 1 M salt is due to conformational changes in the N-terminal region of E1 or to inhibition of trypsin itself. Turn-off rates for E1 appear to be faster in 0.1 M KCl than in 1 M KCl at *trans* pH 7 (*compare* Fig. 6*D* with Fig. 7, before trypsin), and trypsin works well from the *cis* side in 1 M KCl; both of these results argue for the former possibility. As mentioned in the Results, however, higher concentrations of trypsin are required to effect conversion of ThCF channels in 1 M KCl than in 0.1 M KCl, whereas turn-off rates before trypsin are comparable under both salt conditions (*data not shown*), suggesting that trypsin activity itself is inhibited in 1 M KCl.

[4] In making these calculations, it is assumed that the residue is transferred as part of an alpha helix, so that all backbone hydrogen bonds are still intact in the bilayer (Engelman & Steitz, 1981).

than the first, however, since it requires movement of the entire stretch of 386 amino acids found N-terminal to the CNBr(6-1) fragment in order for E1 channels to open.

The possibility that channels formed by each of the four peptides actually differ slightly in structure must be considered. For instance, the most N-terminal membrane-spanning domain that participates in forming the channel walls might be different for each peptide, as depicted in Fig. 10*B*. Such a variation in structure could be the basis for the differences seen in selectivity among the different channels. Results of selectivity experiments with ThCF channels, at symmetric pH 7 before and after *trans* trypsin digestion, however, argue against this possibility. Our results show that the reversal potential of open ThCF channels changes upon exposure to *trans* trypsin while the channels remain open. If the one variable membrane-spanning domain were affecting selectivity, and if this segment were detached from the rest of the channel-forming region by trypsin digestion, then the measured reversal potential would not change until the modified channel had first been turned off and then back on again. This is because trypsin cleavage could only have one of two effects in such a case: (i) it could detach one membrane-spanning region from an open channel and thereby destroy that open channel, but once this destroyed channel had slipped out of the membrane back on the *cis* side, it could turn on again as a "modified", CNBr(6-1)-like channel, or (ii) trypsin cleavage could have no effect on open channels, if interactions among the membrane-spanning domains were to keep channel lumens intact—that is, until the channels were turned off with voltage; after that, these channels would turn back on as "modified" channels with a new reversal potential. Since *trans* trypsin digestion *does* affect the reversal potential of open ThCF channels, the model depicted in Fig. 10*B* is probably incorrect.

Although the effect of glutathione on turn-off rates of E1 and ThCF channels has been quite useful in these investigations, we have not yet determined glutathione's mechanism of action. It is probably not interacting with the lumen itself, since this is assumed to be identical for channels formed by each of the four peptides. Glutathione, a molecule that has one positive and two negative charges at pH's around 4, may be interacting with some of the charged amino acids postulated to be located on the *trans* side of the membrane in the channel's open state. In support of this theory, another negatively charged molecule, glucuronate, also has been found to slow the turn-off rates of E1 and ThCF channels from the *trans* side, but its effect is much less profound.

An important finding of this paper is that the CNBr(6-1) fragment (Asn_{387} through Ile_{522}) forms channels very similar in gating and selectivity properties at low pH to those of whole E1, suggesting that the same molecular structure forms the channel in both instances. The CNBr(6-1) fragment contains 136 amino acids, which imposes some limitations on molecular models which are consistent with the sequence. [If the C-terminal ten amino acids are not essential for channel formation (Ohno-Iwashita & Imahori, 1982), an upper limit of 126 amino acids is placed on the channel-forming segment.] For example, it seems to eliminate the specific models proposed by Guy (1983) and by Davidson, Brunden, Cramer and Cohen (1984), since both of these require amino acids which are upstream of Asn 387. Additional Met mutants which produce new CNBr sites have been made and are now being tested. These impose more severe limitations on the length of the channel-forming peptide, and the implications of these limitations on the possible molecular structures that form the channel will be reported (Q.R. Liu et al., *in preparation*).

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