Membrane Binding of the Colicin E1 Channel: Activity Requires an Electrostatic Interaction of Intermediate Magnitude

S. D. Zakharov, J. B. Heymann, Y.-L. Zhang, and W. A. Cramer
Department of Biological Sciences, Purdue University, W. Lafayette, Indiana 47907 USA

ABSTRACT In vitro channel activity of the C-terminal colicin E1 channel polypeptide under conditions of variable electrostatic interaction with synthetic lipid membranes showed distinct maxima with respect to pH and membrane surface potential. The membrane binding energy was determined from fluorescence quenching of the intrinsic tryptophans of the channel polypeptide by liposomes containing *N*-trinitrophenyl-phosphatidylethanolamine. Maximum in vitro colicin channel activity correlated with an intermediate magnitude of the electrostatic interaction. For conditions associated with maximum activity (40% anionic lipid, I = 0.12 M, pH 4.0), the free energy of binding was $\Delta G \approx -9$ kcal/mol, with nonelectrostatic and electrostatic components, $\Delta G_{nel} \approx -5$ kcal/mol and $\Delta G_{el} \approx -4$ kcal/mol, and an effective binding charge of +7 at pH 4.0. Binding of the channel polypeptide to negative membranes at pH 8 is minimal, whereas initial binding at pH 4 followed by a shift to pH 8 causes only 3–10% reversal of binding, implying that it is kinetically trapped, probably by a hydrophobic interaction. It was inferred that membrane binding and insertion involves an initial electrostatic interaction responsible for concentration and binding to the membrane surface. This is followed by insertion into the bilayer driven by hydrophobic forces, which are countered in the case of excessive electrostatic binding.

INTRODUCTION

The defining event in the functioning of the cytotoxin colicin E1 is the formation of a voltage-gated, highly conductive ion channel in the inner membrane of an *Escherichia coli* cell (in vitro single-channel conductance $> 10^6$ ions/s in 0.1 M salt) (Bullock et al., 1983; Cleveland et al., 1983). This depolarizes the cytoplasmic membrane (Gould and Cramer, 1977), resulting in inhibition of active transport (Dankert et al., 1980) and subsequent rapid depletion of ATP and internal K^+ (Phillips and Cramer, 1973; Kopecky et al., 1975; Cramer et al., 1992) and possibly phosphate (Guihard et al., 1993) levels. Multiple steps precede the dissipative events in the cytoplasmic membrane, including binding of the central domain of the colicin to the outer membrane vitamin B_{12} receptor, BtuB (Brunden et al., 1984); translocation across the cell envelope requiring the N-terminal domain that interacts with the *tol* translocation network (Webster, 1991); binding of the C-terminal, channel-forming domain (Dankert et al., 1982) to the cytoplasmic membrane, which is thought to occur with some unfolding at the tertiary structure level (Zhang and Cramer, 1992; Schendel and Cramer, 1994); and partial insertion into the membrane.

As the penultimate step in the functioning of colicin E1, the binding of the protein to the membrane is an essential event that must be related to its activity. The mechanism by which water-soluble toxins such as colicins insert into membranes is a fundamental unsolved problem in membrane-protein interactions. A role of electrostatic (Davidson et al., 1985; Kayalar and Düzgünes, 1986; Xu et al., 1988) and hydrophobic (Shin et al., 1993) interactions in the binding of the channel to the membrane at acidic pH in vitro has been described for the colicin E1 channel, and of electrostatic interactions in vitro (González-Mañas et al., 1993; Lakey et al., 1994) and in vivo (van der Goot et al., 1993) for the channel domain of colicin A. The quantitative parameters of the equilibrium binding reaction of the colicin E1 channel with membranes have recently been characterized (Heymann et al., 1996).

In the present study, investigations on the interaction of the colicin E1 channel-forming domain with membranes have been extended to correlate binding parameters with channel-forming activity. The binding parameters were obtained by the nonradiative energy transfer of the intrinsic tryptophan (Trp) fluorescence to a membrane-bound trinitrophenyl-phosphatidyl-ethanolamine (TNP-PE) quencher, using the binding analysis developed by Heymann et al. (1996). This approach involves the titration of protein with TNP-PE-containing artificial vesicles of defined anionic

Received for publication 3 January 1996 and in final form 21 March 1996.

Address reprint requests to Dr. William Cramer, Department of Biological Sciences, Purdue University, West Lafayette, IN 47907. Tel.: 317-494-4956; Fax: 317-496-1189; E-mail: wac@bilbo.bio.purdue.edu.

Abbreviations: DMG, dimethylglutaric acid; DOPC, DOPE, DOPG, DOPS, 1,2-dioleoyl-phosphatidylcholine, phosphatidylethanolamine, phosphatidylglycerol and phosphatidylserine; FRET, fluorescence resonance energy transfer; ΔG, ΔG_{el}, ΔG_{nel}, total, electrostatic, nonelectrostatic free energy of binding; I, ionic strength; K_d, protein-membrane dissociation constant; LUV, large unilamellar vesicles; n, lipid:protein binding stoichiometry; P178, P190, thermolytic, engineered C-terminal colicin E1 channel polypeptides; Ψ_o, membrane surface potential; TNBS, trinitrobenzenesulfonic acid; TNP-PE, N-trinitrophenyl-phosphatidylethanolamine; Trp, tryptophan; x_a, accessible lipid; x_-, fraction of anionic lipid in membrane; $\bar{v}_L$, specific lipid molar volume; z_{eff}, effective binding charge.

Dr. Zhang's present address is Jules Stein Eye Institute, Department of Chemistry, University of California–Los Angeles, Los Angeles, CA 90095-7008.

Dr. Zakhavov's permanent address is Institute of Soil Science and Photosynthesis, Russian Academy of Sciences, Puschino, Russia.

0006-3495/96/06/2774/10 $2.00

and neutral lipid composition, through the decrease in protein Trp fluorescence.

It was concluded from the data that binding of the colicin channel polypeptide to and insertion into the membrane involves 1) an initial electrostatic interaction that is responsible for the binding of the positively charged polypeptide to the negatively charged membrane surface at the acidic pH used in in vitro experiments, 2) followed by a hydrophobic interaction that is associated with a deeper insertion of segments of the protein into the interfacial layer and perhaps across the hydrophobic core of the membrane bilayer. It is proposed that the requirement for maximum activity of an electrostatic interaction of intermediate strength reflects a balance of forces associated with the transition from a surface-bound state to an inserted state. Preliminary accounts of these studies have been presented (Zhang et al., 1994; Zakharov et al., 1995).

MATERIALS AND METHODS

Preparation of colicin E1 and its channel polypeptides

1) Colicin E1 was purified as previously described (Zhang and Cramer, 1992). 2) The C-terminal channel polypeptide with a ragged N-terminus, 174, 177, or 178 residues in length, P178, was prepared by proteolysis of the intact colicin E1 with thermolysin (Bishop et al., 1985). 3) The engineered colicin E1 C-terminal channel polypeptide, P190, was expressed from pSKHY, a plasmid with the colicin channel domain under control of the colicin E1 promoter (Elkins et al., 1994), and purified as previously described (Zhang and Cramer, 1992). The modified Lowry assay (Peterson, 1977) was used to determine the protein concentration.

Preparation of large unilamellar vesicles

Large unilamellar vesicles (LUVs) were prepared by an extrusion procedure (Hope et al., 1985). Aliquots of synthetic phosphatidylglycerol (DOPG), 1,2-dioleoyl-phosphatidylcholine (DOPC) (Avanti Polar Lipids, Alabaster, AL), and TNP-PE (Sigma, St. Louis, MO) (10% w/w ≡ 8.4 mol %) in chloroform were mixed to obtain the desirable molar ratios in a total of 10 mg lipid. The glass surface of a test tube was coated with lipids by evaporation of the solvent under a stream of nitrogen during vortexing, and traces of solvent were removed under vacuum for 3–4 h. The lipid was resuspended in 1 ml buffer (0.1 M KCl, 10 mM dimethylglutaric acid (DMG), pH 5.0) by vortexing under nitrogen and subjected to 10 freeze-thaw cycles. The vesicles were extruded 10 times through a double polycarbonate filter with pore size 0.1 μm (Nuclepore, Costar, Cambridge, MA) to obtain extruded LUVs. The Bartlett assay for phosphorus was carried out according to the wet digestion method of New (1990). Accessible lipid, x_a, was measured by derivatization of phosphatidylethanolamine lipid (incorporated in LUVs in place of TNP-PE) by trinitrobenzenesulfonic acid (New, 1990). LUVs prepared as for the binding assays consistently showed fractional lipid accessibilities of $x_a = 0.52 \pm 0.03$.

In vitro channel-forming activity

In vitro activity of the channel polypeptide toward liposomes was measured by the rate of channel-induced chloride efflux, detected with a chloride-specific electrode (Peterson and Cramer, 1987), from extruded LUVs further enlarged by 10 extra freeze-thaw steps.

Fluorescence measurements

Binding of protein was assayed in 1-cm path length cuvettes at 23°C by quenching of Trp fluorescence with TNP-PE-containing vesicles using a SLM 8000C photon-counting spectrofluorimeter (SLM Instruments, Urbana, IL). The samples were magnetically stirred. Excitation and emission wavelengths were calibrated, using the 296.7 and 365.0 nm Hg lines of a low pressure mercury lamp. Emission spectra were acquired with excitation at 280 nm to obtain a complete Trp emission spectrum (which includes excitation of tyrosine residues, although their contribution to the emission spectrum is negligible) and a bandpass of 4 nm for both beams. Kinetic and titration data were recorded through excitation of the three intrinsic tryptophans at 293 nm (which minimizes excitation of tyrosine residues), and emission was measured at 340 nm, with half-band widths of 4 nm and 8 nm, respectively. The signal was corrected for the variation of excitation intensity by using a reference channel. Because the titration involved measurements of highly quenched low signal levels, polarization optics was not used. However, the existence of polarization artifacts was shown to be insignificant at low levels of quenching by setting the emission beam polarizer at 54.7° relative to the vertical polarization axis of the exciting light (Lakowicz, 1983). The R_o value for 50% of fluorescence resonance energy transfer from the Trp residues of the colicin E1 channel domain to TNP was determined to be 27 ± 1 Å by A. Szabo and J. Brennan from the overlap integral (personal communication) (Lakowicz, 1983). This large R_o value, which is comparable to the mean dimension, ~40 Å, of the channel polypeptide, implies that quenching of the Trp fluorescence is not sensitive to the precise orientation of the channel polypeptide and the exact positions of the three Trp residues at the membrane surface.

Titration of protein with membrane vesicles

LUVs containing 10% TNP-PE and variable amounts of DOPG and DOPC were used to assay the lipid affinity of the channel polypeptide. Protein (4 μg/ml ≡ 192 nM for P190; 206 nM for P178; 11 μg/ml ≡ 196 nM for colicin E1) was titrated with small aliquots of LUV suspension (2–8 μl of 0.5–5 mg/ml additions) to a final concentration of 20–500 μM. All titration points were recorded at equilibrium, 5–10 min after each addition of vesicles. Liposomes containing different amounts of the anionic lipid DOPG and the neutral lipid DOPC were used to assay the membrane-binding affinity of the channel polypeptide as a function of pH, ionic strength, and membrane surface charge density.

Parameters for the binding of a protein to a membrane

The binding of the protein to a membrane was described with a bimolecular interaction model (Hille et al., 1981). This approach assumes that free protein, P_f, binds to a membrane, interacting with a unit of multiple (n) lipid molecules, $(L_f)_n$, to form a complex, P_b:

$$P_f + (L_f)_n \overset{K_d}{\rightleftharpoons} P_b.$$

The dissociation constant, K_d, for the interaction of the polypeptide with one binding unit of n lipid molecules on the vesicle surface is

$$K_d = \frac{[L_f][P_f]}{n[P_b]}, \quad (1)$$

where n is the lipid:protein stoichiometry, $[P_f]$ and $[P_b]$ are the free and bound protein concentrations, and $[L_f]$ is the "free" lipid concentration (i.e., not bound to polypeptide). The relevant component of the fluorescence

signal arises from the fraction of protein not bound to the vesicles (see Heymann et al., 1996, for the derivation):

$$f_f \equiv \frac{[P_f]}{[P]} = \frac{1}{2}\left[1 - \frac{[L]}{n[P]} - \frac{K_d}{[P]} + \sqrt{\left(1 - \frac{[L]}{n[P]} - \frac{K_d}{[P]}\right)^2 + 4\frac{K_d}{[P]}}\right], \quad (2)$$

where [L] and [P] are the total lipid and protein concentrations, respectively.

The energy involved in the transfer of one protein molecule from one infinite phase (aqueous solution) to another infinite phase (the membrane) can be described in a concentration-independent formalism by a partition coefficient, K_b, in terms of the reactant volumes:

$$K_b = \frac{V_{Pb}/(V_L + V_{Pb})}{V_{Pf}/(V_{aq} + V_{Pf})} \approx \frac{V_{Pb}/V_L}{V_{Pf}/V_{aq}}, \quad (3)$$

where V_{Pb}, V_{Pf}, V_L, and V_{aq} are the partial volumes associated with bound and free polypeptide, total lipid, and total aqueous volume, respectively. The right-hand expression applies for $V_{Pf} \ll V_{aq}$ and concentrations of protein such that $V_{Pb} \ll V_L$. In this case, K_b can be expressed in terms of K_d and n, under 1) the condition of excess lipid, $[L_f] \approx [L]$, 2) the assumption that the ratio of protein volumes is proportional to the ratio of their concentrations, i.e.,

$$\frac{V_{Pb}}{V_{Pf}} \approx \frac{[P_b]}{[P_f]} = \frac{[L_f]}{nK_d}$$

(the equality from Eq. 1), 3) with the use of the relation $V_L = \bar{v}_L [L] V_{aq}$ ($\bar{v}_L$ is the specific lipid molar volume, ~0.8 liter/mol), and 4) by substitution into Eq. 3:

$$K_b = \frac{1}{\bar{v}_L n K_d}. \quad (4)$$

The bimolecular interaction and partitioning approaches to protein-membrane interactions therefore converge to the same solution for very dilute protein. The total binding energy is then

$$\Delta G = -RT \ln K_b = RT \ln(\bar{v}_L n K_d). \quad (5)$$

The free energy of protein binding to the membrane has an electrostatic component dependent on the vesicle surface potential, ψ_o, and the effective interacting charge, z_{eff}, as well as a component independent of electrostatic binding. Equation 5 can then be expressed in terms of its electrostatic ($\Delta G_{el} = z_{eff} F \psi_o$) and nonelectrostatic components ($\Delta G_{nel} = -RT \ln K_{nel}$):

$$\Delta G = z_{eff} F \Psi_o - RT \ln K_{nel}, \quad (6)$$

where K_{nel} is the nonelectrostatic partition coefficient for the protein between membrane and bulk phase at $\psi_o = 0$. The surface potential, ψ_o, was calculated from Gouy-Chapman theory (McLaughlin, 1989; Seelig et al., 1993; Heymann et al., 1996) by numerically solving the following simultaneous equations for the surface charge density, σ, and ψ_o, using the program Mathcad 3.1 (MathSoft, Cambridge, MA):

$$\sigma = \frac{e\_x\_}{A_L[1 + \sum_X K_{XL}[X^z] e^{-(zF\Psi_o/RT)}]} \quad (7)$$

$$\sigma = -\sqrt{2\epsilon\epsilon_o RT \sum_X [X^z](e^{-(zF\Psi_o/RT)} - 1)}, \quad (8)$$

where $e\_$ is the elementary charge (-1.6×10^{-19} coulombs), $x\_$ is the fraction of negative lipid in the membrane, $A_L = 65$ Å^2 (Lantzsch et al., 1994) is the lipid surface area, X^z refers to an ion with charge z (Na^+, choline$^+$, Ca^{2+}, and Cl^- were considered where appropriate), and the cation-negative lipid association constants K_{XL} are $K_{NaL} = 0.6$ M^{-1} (Seelig et al., 1993), $K_{CaL} \approx 25$ M^{-1}, and $K_{ChL} \approx 4$ M^{-1} for sodium, calcium, and choline, respectively (the latter two were estimated from the best fit to the experimental binding data).

RESULTS

In vitro channel activity as a function of pH, anionic lipid content, and calcium

The channel activity of a proteolytically (thermolysin) derived C-terminal 174–178 residue polypeptide, P178, was determined with DOPC:DOPG membrane vesicles as a function of anionic lipid (DOPG) content ($x\_$) and pH (Fig. 1 *A*). LUV was enlarged by multiple freeze-thaw cycles to encapsulate sufficient solute (Cl^-) to allow detection of efflux induced by the addition of P178 (Peterson and Cramer, 1987). P178 exhibited very low activity ($<10^3$ ions/channel/s) toward neutral lipid vesicles (100% DOPC), showed a low activity with vesicles containing 10% PG ($x\_ = 0.1$), and maximum activity (~10^4 ions/channel/s) with 30% PG vesicles in the pH range 3.2–3.8 (Fig. 1 *A*). Increasing the anionic lipid content to $x\_ = 0.5$ lowered the activity, but shifted the range for maximum activity to pH 4.4, with an apparent peak at pH 4.2. Channel activity is further decreased in vesicles with $x\_ > 0.5$, and the pH optimum shows a further alkaline-directed shift (Fig. 1 *A*).

In the membrane-binding assay used in this study (see below), anionic TNP-PE was incorporated into the LUVs. The anionic lipid content of the vesicles used for fluorescence quenching measurements was then equal to the PG content plus a contribution of 10% from the TNP-PE. This presence of the anionic TNP-PE in liposomes was found to affect activity in the same way as an equivalent amount of DOPG, exhibiting the same variation in activity and shift in pH optimum (data not shown). Similarly, the anionic lipid DOPS could be substituted for DOPG (data not shown), implying that the formal lipid headgroup charge, independent of its chemical identity, is the major lipid headgroup parameter that influences channel activity.

The low activities found for vesicles with both a low and a high anionic lipid content imply that either a lack of negative surface charge, which leads to weak binding, or an excessive surface charge decreases the ability of the channel polypeptide to properly bind to, or insert into, the membrane and form a functional ion channel. A reciprocal relationship between negative membrane surface charge and net positive charge of the channel polypeptide is implied by 1) the shift of maximum channel activity toward higher pH values in vesicles with higher PG content (e.g., $x\_ = 0.3 \rightarrow 0.5 \rightarrow 0.7$) (Fig. 1); and 2) the shift of maximum activity to lower pH by Ca^{2+} (Fig. 1 *B*), which binds to the phospholipid headgroups and thereby weakens the electrostatic interaction between the channel polypeptide and the membrane

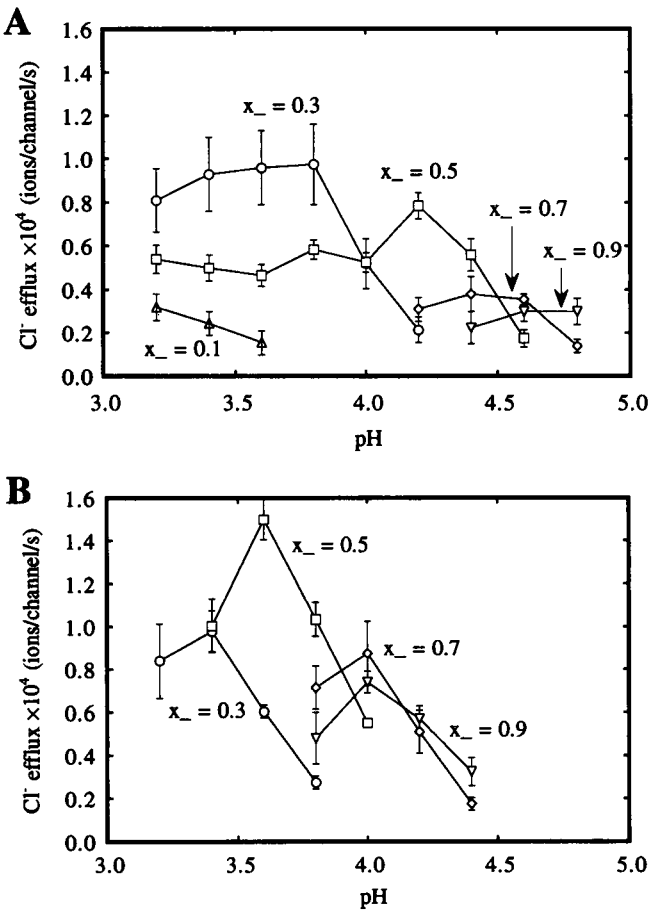

FIGURE 1 (*A*) pH dependence of solute (Cl^-) efflux activity of colicin E1 channel polypeptide, P178, as a function of anionic lipid content (x_-) in freeze-thaw enlarged LUVs. DOPG:DOPC vesicles were added to a final concentration of 100 μg/ml in 15 ml (20 mM Na-DMG, 50 mM choline-NO_3, 50 mM $NaNO_3$, I = 0.12 M) and equilibrated for 2–4 min. Valinomycin was added to a final concentration of 10 nM 20–30 s before Cl^- efflux was induced by the addition of 0.3–1.3 nM P178. Total Cl^- encapsulated in the vesicles was measured by its release with 0.1% Triton X-100. (*B*) Effect of 2 mM calcium nitrate on the pH and anionic lipid dependence of chloride efflux. Activities of $<10^3$ ions/channel/s could not be measured reproducibly, and these cases were omitted from the graph. △, x_- = 0.1; ○, x_- = 0.3; □, x_- = 0.5; ◇, x_- = 0.7; ▽, x_- = 0.9.

surface. In the presence of 2 mM Ca^{2+}, there is a sixfold decrease in channel activity observed at pH 4 (with x_- = 0.4 and I = 0.12 M; Table 1), and a shift of the high pH threshold for activity from pH 3.8 to 3.4, pH 4.2 to 3.6, and from approximately pH 4.5 to 4.0 using vesicles with x_- = 0.3, 0.5, and 0.7, respectively (Fig. 1 *B*). This implies that an intermediate strength of the electrostatic interaction between channel polypeptide and the membrane surface is required for optimal channel activity.

Other observations (not shown) that illustrate the reciprocal relationship between parameters involved in the initial electrostatic interaction of the colicin channel polypeptide with the membrane are 1) an acid-directed shift of 0.2–0.3 units in the high-pH threshold of channel activity when the ionic strength is increased from 0.12 to 0.17 M (x_- = 0.3–0.5); 2) an acid-directed shift of 0.2 units in the pH optimum for channel activity measured with the P190 channel polypeptide, which has two additional negative charges (N-terminal sequence: METAENNLLNSQIKDAV...), compared to the optimum measured with P178 (N-terminal sequence: IKDAV... ; potentially charged residues: 24 Lys, 1 Arg, 2 His, 15 Asp, and 7 Glu).

Membrane binding parameters of the channel polypeptide

The parameters that describe the equilibrium binding of proteins to membranes are the dissociation constant, K_d, and the lipid:protein stoichiometry, n, determined by titration of the thermolytic channel polypeptide (P178) Trp fluorescence with liposomes in which the quencher TNP-PE has been incorporated to a level of 10% (Heymann et al., 1996). Titrations as a function of pH (Fig. 2 *A*), ionic strength (Fig. 2 *B*), and anionic lipid content, x_- (Fig. 2 *C*), with 0.1 μm extruded LUVs containing 10% TNP-PE imply a major role for electrostatic interactions in the binding-insertion process of P178. As a function of decreasing pH (Fig. 2 *A*), which causes an increase in net positive charge on the protein, decreasing ionic strength (Fig. 2 *B*) and increasing anionic lipid content (x_-, Fig. 2 *C*), there is a monotonic 1) decrease in dissociation constant, K_d, and 2) decrease in the number of lipid molecules, n, associated with the binding of the polypeptide. For LUVs at I = 0.3 M with x_- = 0.4, K_d and n decrease monotonically to 3.5 nM and 61 at pH 3.5 (Fig. 2 *A*).

Because choline was used as cation in in vitro channel-forming assays (Peterson and Cramer, 1987), the binding of P178 to LUVs with x_- = 0.4 (30% DOPG and 10% TNP-PE) was determined under the conditions used for activity assays (pH 4.0, I = 0.12 M, 20 mM Na-DMG, 50 mM choline-NO_3, 50 mM $NaNO_3$). The binding parameters were K_d = 7.5 ± 1.9 nM and n = 55 ± 6 mol/mol, with the dissociation constant most affected compared to the K_d = 1.9 nM without choline (Table 1). This suggested that the positively charged choline effectively shields the negative membrane surface charge, consistent with the electrostatic interaction described above.

The absolute value of K_d is smaller and the binding affinity greater for P178 compared to the binding parameters for the longer engineered P190 polypeptide (Table 1), which has two additional negatively charged Glu residues at its N-terminus. The binding of the entire 522 residue colicin E1 protein under the same conditions is characterized by a smaller K_d value (1.3 nM) and higher affinity, and a much larger n value (211 versus 50–57) than found for the channel polypeptides. Binding of the P293 colicin fragment containing receptor and channel domains is characterized by an n of intermediate value (Heymann et al., 1996). These data show that the TNP-PE fluorescence quenching method is able to resolve small changes in binding parameters associated with variants of the same polypeptide.

Reversibility of the binding of P178 to membranes

If the predominant component of binding of the channel domain to charged membranes is electrostatic, it should be possible to reverse its binding by increasing the pH and/or ionic strength (Davidson et al., 1985; Xu et al., 1988). A small increase in the fluorescence (3–10%) of P178 is observed after an alkaline-directed pH shift from 4 to 7.5–8.0 (Fig. 3 *A*). The alkaline condition immediately stops any further binding. The fluorescence increase is small for P178 whether the pH shift occurs 5 s or 5 min after initiation of binding, suggesting that the electrostatic binding is accompanied by an additional specific nonelectrostatic (e.g., hydrophobic) component. A somewhat larger reversal of binding (~15%) of the 187 residue tryptic channel polypeptide induced over 30 min by alkali and increased ionic strength was previously observed through intervesicle transfer in the presence of exogenous liposomes (Xu et al., 1988).

A pronounced time dependence of the reversal of the fluorescence quenching associated with binding was observed with the intact 522 residue colicin E1 molecule. The initial time course of fluorescence quenching was more rapid with colicin than with channel polypeptide, but a substantial reversal of the quenching (> 50%) could be obtained with a pH jump 5 s (Fig. 3 *B, curve a*) after initiation of binding at pH 4. The extent of the reversal decreased markedly after longer (1–4 min) incubation times at pH 4 (Fig. 3 *B, curves b, c*). This implies that, after fast electrostatic interaction with the membrane, the rate of insertion of the membrane-active domain of the whole colicin into the membrane is markedly slower than for the channel polypeptide.

Many common water-soluble proteins bind electrostatically to LUVs at pH 4.0 (e.g., lysozyme; Heymann et al., 1996). The fluorescence quenching and binding of the soluble proteins lysozyme, carbonic anhydrase, and cytochrome *c* (Fig. 3 *C, traces a–c*, respectively), are distinguished from those of colicin and its channel polypeptide fragments by an almost complete reversal of the quenching by an alkaline-directed pH shift. This is consistent with the peripheral, purely electrostatic mode of binding of such proteins demonstrated by the lack of quenching by LUVs containing the deeply embedded bromine quencher in 1,2-distearoyl(9,10-dibromo)phosphatidylcholine (Heymann et al., 1996). It also implies, at least for the large increases of fluorescence, that the increase in Trp fluorescence in the pH jump experiments is an indicator of reversal of binding rather than of conformational change.

Membrane binding energy of the colicin E1 channel polypeptide

Consistent with Eq. 6, a linear relationship is obtained between the surface potential and the total free binding energy, ΔG, by using the binding data of Fig. 2, *B* and *C* (Fig. 4). The value of ΔG for $\psi_o = -30$ mV is approximately -9.5 ± 0.2 kcal/mol. By extrapolation to zero surface potential, the contribution of nonelectrostatic interactions to the total binding energy was found to be $\Delta G_{nel} = -5.0$ kcal/mol (Fig. 4), and the free energy of electrostatic binding is $\Delta G_{el} = -4.5$ kcal/mol. In the presence of choline, the binding energy decreases to $\Delta G = -8.7 \pm 0.2$ kcal/mol because of the decrease in surface potential to $\psi_o = -23$ mV.

The effective number of charges, z_{eff}, involved in the electrostatic binding was calculated from $\Delta G_{el} = z_{eff} F \psi_o$ (see Eq. 6) for each pH point shown in Fig. 2 *A* (Fig. 5). The effective binding charge is $z_{eff} = 7.0$ at pH 4.0. Because the expected pKs of the Asp and Glu carboxylates fall in the range of 3.9–4.4 (Nozaki and Tanford, 1967), the effective charge was fit to a Henderson-Hasselbalch type equation, yielding an average pK of 4.0 for P178 (Fig. 5), with an asymptotic maximum effective binding charge at low pH, $z_{effm} = 14.1$, at pH values of <3. Near the midpoint of the curve, the z_{eff} has a large dependence on pH, and is 10.3 and 2.3 at pH 3.5 and 4.5, respectively.

Strength of electrostatic interaction associated with maximum activity

The shift of the optima for channel activity to higher pH, resulting from increased anionic lipid content or decreased Ca^{2+} concentration (Fig. 1), corresponds to oppositely directed changes in net charge of the polypeptide and the

TABLE 1 Comparison of the binding parameters *n* and K_d, binding energy ΔG, and effective charge z_{eff} with Cl^- efflux activity of colicin E1 and its C-terminal polypeptides, P178 and P190

Protein	Ca^{2+} (mM)	*n* (mol/mol)	K_d (nM)	ΔG (kcal/mol)*	z_{eff}	Cl^- efflux × 10^4 (ions/channel/s)#
P178	0	50 ± 4	1.9 ± 0.4	−9.7	7.0	1.30 ± 0.30
	2	71 ± 4	5.5 ± 1.1	−8.9	7.0	0.20 ± 0.05
	5	123 ± 7	12.5 ± 3.7	−8.0	7.0	0.10 ± 0.02
P190	0	57 ± 3	3.1 ± 0.4	−9.3	6.4	0.70 ± 0.30
Colicin E1	0	211 ± 10	1.3 ± 0.2	−9.1	5.9	1.90 ± 0.65

*Binding parameters were measured with 30% DOPG:60% DOPC:10% TNP-PE LUVs at pH 4.0, 20 mM Na-DMG, 100 mM NaCl, $I = 0.12$ M, $\Psi_o = -29.8$ mV; number of trials = 3–5.

#Cl^- efflux activity was measured with 40% DOPG:60% DOPC LUVs at pH 4.0 in 20 mM Na-DMG, 50 mM choline-NO_3, 50 mM $NaNO_3$, $I = 0.12$ M, $\Psi_o = -23.3$ mV; number of trials = 3–6.

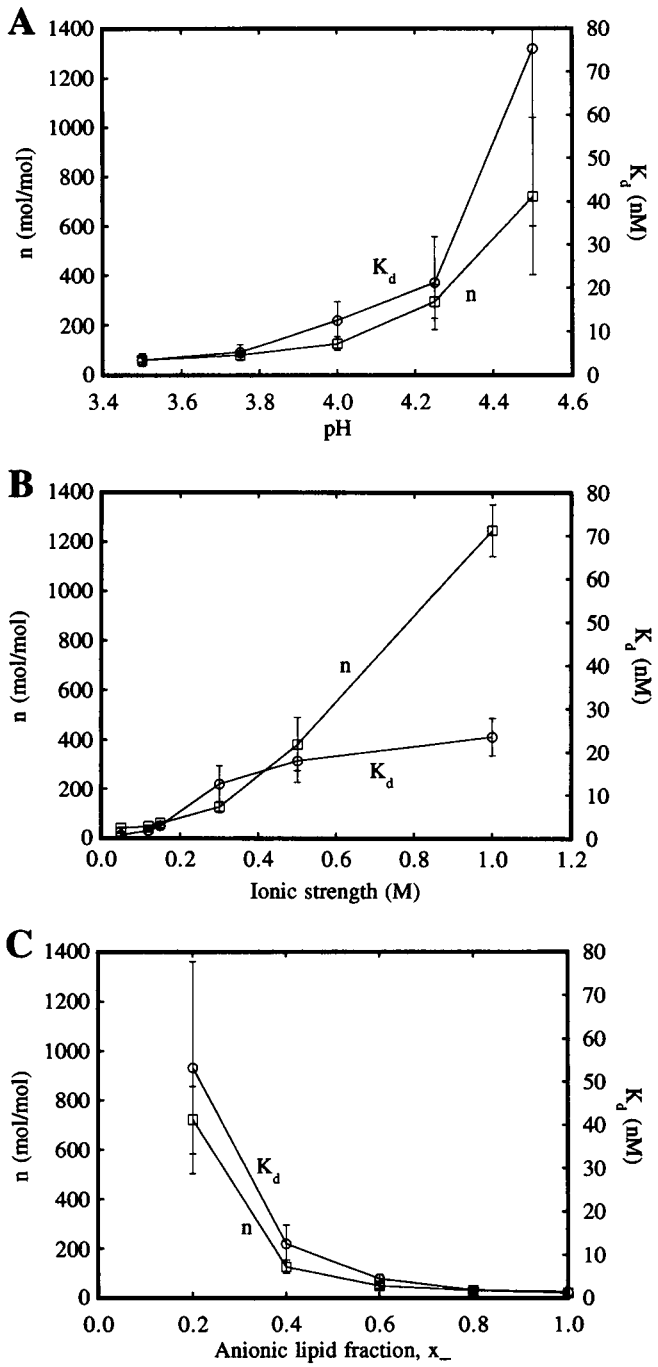

FIGURE 2 Dependence of the parameters K_d (○) and n (□) for binding of P178 to LUVs on (*A*) pH, (*B*) ionic strength (*I*), and (*C*) anionic lipid content (x_-), determined by titration of 0.21 μM protein with TNP-PE LUV in 20 mM DMG-NaOH, with standard conditions: 0.28 M NaCl (I = 0.3 M), pH 4.0, and 30% DOPG:60% DOPC:10% TNP-PE (x_- = 0.4). Ionic strength was varied by addition of NaCl (*B*); anionic lipid content, x_-, was varied through the DOPG:DOPC ratio (*C*).

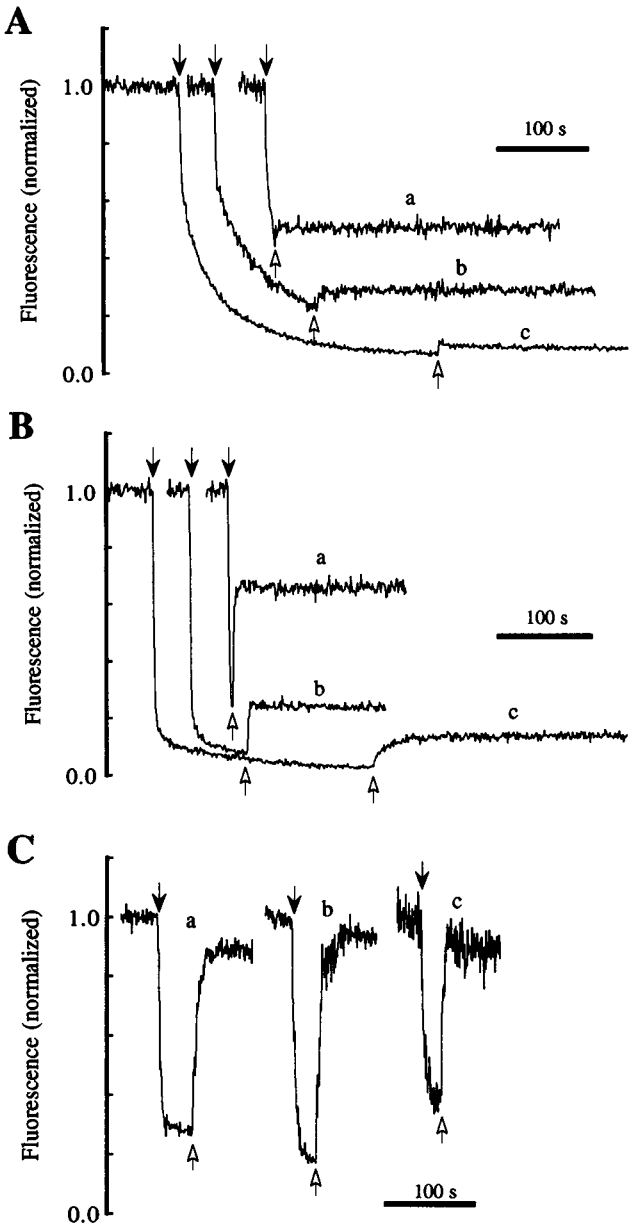

FIGURE 3 (*A*) Limited release from LUVs of channel polypeptide, initially bound at pH 4.0, by a shift to pH 7.5–8.0, assayed by reversal of fluorescence quenching. Solid arrows indicate the addition of aliquots of LUVs (each 100 μM lipid) to 0.2 μM P178 (*a, c*) or P190 (*b*) in 20 mM DMG, pH 4.0, 0.2 M NaCl. Open arrows indicate addition, 10 s (*a*), 2 min (*b*), or 5 min (*c*) after the addition of LUVs, of 20 μl 0.3 M Tris-3 M NaOH to yield a final pH of 7.5–8.0. (*B*) Time-dependent decrease in the release of the 522 residue colicin E1 (0.2 μM) from LUVs by the addition of alkali (pH 4→8) at 5 s (*a*), 70 s (*b*), or 250 s (*c*) after the addition of LUVs. Other conditions are as in *A*. (*C*) Release of (*a*) lysozyme, (*b*) carbonic anhydrase, and (*c*) apocytochrome *c* by alkali (pH 4→8) added 40 s, 30 s, and 25 s after the addition of a quenching amount of TNP-PE LUV to a final concentration of 150 μM (*a*), 100 μM (*b, c*) lipid. Other conditions are as in *A*, except that NaCl concentration = 0.1 M.

membrane surface. The consequences of the reciprocal charge changes are 1) the electrostatic binding energy, $\Delta G_{el(max)}$, associated with maximum activity (maximum values, A_{max}, from Fig. 1 *A* and Table 1, line 1) does not increase monotonically with increasing anionic lipid content, x_- (Fig. 6). The magnitude of $\Delta G_{el(max)}$ does increase to a maximum of −4.0 kcal/mol (shown as a minimum in Fig. 6) as x_- increases from 0.1 to 0.3–0.5 and the pH for

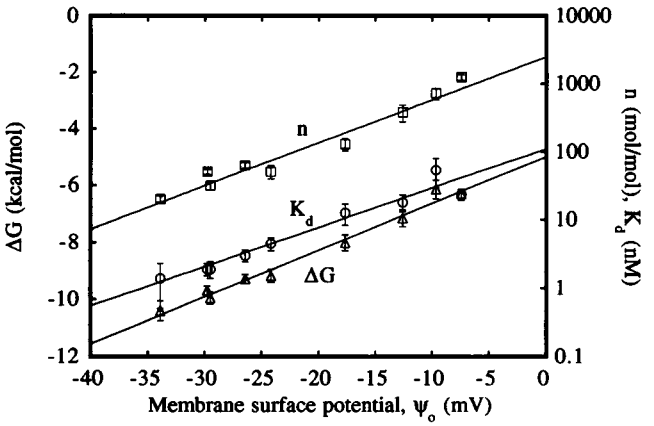

FIGURE 4 Thermodynamic parameters, K_d (○) and n (□), contribute equally to the free energy for binding of P178, ΔG (△), which is composed of electrostatic ($\Delta G_{el} = z_{eff}F\psi_o$) and nonelectrostatic ($\Delta G_{nel}$ determined from the $\psi_o = 0$ intercept) components, with an effective binding charge $z_{eff} = 7.0$ at pH 4.0. Data were taken from Fig. 2, *B* and *C*, and the surface potential was calculated from Gouy-Chapman theory (Seelig et al., 1993; Heymann et al., 1996). The fitted functions are: $n = 110e^{0.13\,\psi_o}$, $r = 0.80$, $K_d = 2500e^{0.15\,\psi_o}$, $r = 0.98$, $\Delta G_{el} = 0.16\psi_o - 5.0$, $r = 0.99$.

maximum activity increases from 3.2 to 4.0. However, the magnitude of $\Delta G_{el(max)}$ decreases as x_- is increased further and pH_{max} increases. 2) The largest value of $\Delta G_{el(max)}$ of −4 kcal/mol (pH = 4.0, $I = 0.12$, and $x_- = 0.4$) is much less than the highest electrostatic binding energy for tight binding conditions at low pH and high negative lipid content (pH 3.4, $I = 0.12$, $x_- = 0.9$) of $\Delta G_{el} = -12$ kcal/mol (calculated for extreme values of the pH and x_- parameters

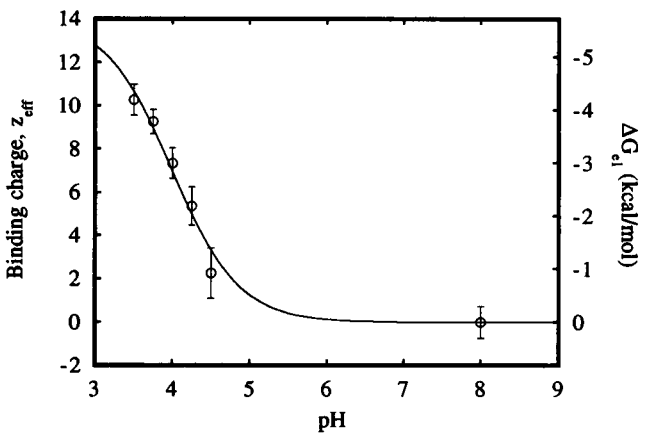

FIGURE 5 Effective binding charge, z_{eff}, calculated from the membrane binding energy of P178 at different pH values (Fig. 3 *A*) and fit to a Henderson-Hasselbalch-type equation. Assuming that the titrable groups (such as the carboxylates) on the protein fall within a narrow range of pK values, a single, average pK could be applied:

$$z_{eff} = \frac{z_{effm}}{1 + 10^{(pH-pK)}} = \frac{14.1}{1 + 10^{(pH-4.0)}},$$

where z_{effm} is the maximum number of interacting charges (e.g., at pH<3) and the correlation index for the fit, $r = 0.99$. $I = 0.3$M; $x_- = 0.4$; $\psi_o = -17.7$ mV.)

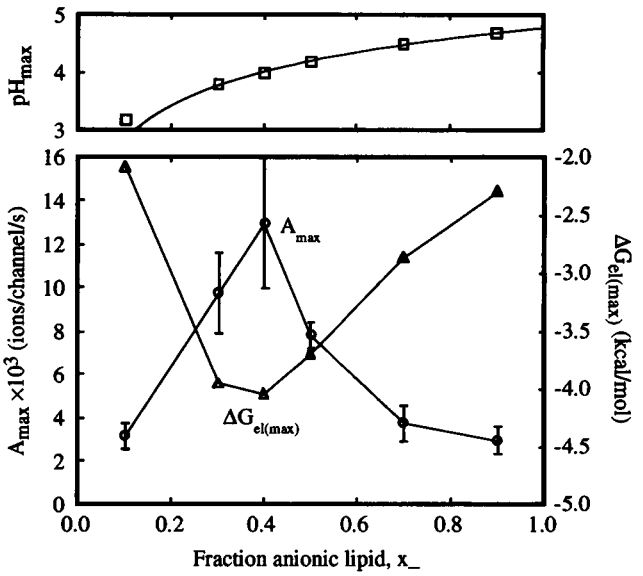

FIGURE 6 Maximum Cl^- efflux activity, A_{max} (○), and the associated pH (pH_{max}, □) and electrostatic binding energy, ($\Delta G_{el(max)}$, △), at which maximum activity was observed as a function of anionic lipid content, x_-. The membrane surface potential, ψ_o, was derived from Gouy-Chapman theory (Seelig et al., 1993; Heymann et al., 1996) for the conditions of the Cl^- efflux experiments, and the electrostatic energy was calculated from $\Delta G_{el} = z_{eff}F\psi_o$, using the pH titration equation in Fig. 5 to determine the effective binding charge, z_{eff}. The empirical relationship between pH_{max} and x_- is $pH_{max} = 4.8 + 0.83\ln x_-$ ($r = 0.99$).

considered here). Thus, it can be seen that maximum channel activity is associated with an intermediate, not a maximum value of the electrostatic binding energy.

DISCUSSION

Details of the electrostatic interaction: the protein net charge

It was suggested from the structure of the colicin A channel domain that a ring of eight lysines around the hydrophobic hairpin might be responsible for docking to the membrane (Parker et al., 1989). The acid titration of the carboxylates unshields the positive charge of the protein that could respond to the membrane surface potential and participate in the initial electrostatic binding. The implication that specific positively charged groups are involved in the binding is weakened by the large maximum effective binding charge observed for P178, $z_{effm} = 14.1$ (Fig. 5), equivalent to half of the 28 basic groups in the molecule. Furthermore, the average pK of 4.0 determined for the binding of P178 is typical for acidic residues. At the membrane surface, the surface potential, $\psi_o = -18$ mV (for $x_- = 0.4$, $I = 0.3$ M; conditions used in the data contributing to Figs. 2 *A*, 5), causes a ΔpK shift ≈ +0.3, yielding an effective pK ≈ 4.3. Therefore, carboxylates close to the lipid headgroups should be protonated to a larger extent, further favoring interaction of the basic protein with the negative surface. The engineered P190 channel polypeptide has two additional car-

boxylates at its N-terminus compared to P178, with a correspondingly lower effective binding charge, $z_{eff} = 6.4$, at pH 4.0 (Table 1). The whole colicin E1 shows a $z_{eff} = 5.9$ at pH 4.0, which is not markedly different from that of the channel polypeptides.

Nonreversible binding

The small extent of the reversal of fluorescence quenching by TNP-PE-containing membranes of the channel polypeptide that is caused by an alkaline-directed pH shift (Fig. 3 *A*), compared to the reversal seen with a variety of water-soluble proteins (Fig. 3 *C*), implies that nonelectrostatic, possibly hydrophobic, interactions are also an important factor in the time interval after the initial contact and binding at low pH. A role for hydrophobic interactions in the membrane binding of colicin is suggested by studies of spin-labeled colicin E1 channel polypeptide added to 12 carbon PC vesicles (Shin et al., 1993).

Taking into account the ~15 kcal/mol loss in one translation and two rotational degrees of freedom on binding to a membrane (Janin and Chothia, 1978; Heymann et al., 1996), the actual ΔG_{nel} is ~ -20 kcal/mol. Engelman and Steitz (1981) calculated a larger hydrophobic binding energy, ~ -60 kcal/mol, for the insertion of a hydrophobic hairpin into the lipid bilayer. The explanation for this disparity may be that the environment of the C-terminal helical hairpin in its initial state before binding to the membrane is the hydrophobic interior of the channel polypeptide in solution (Parker et al., 1992; Elkins et al., 1995).

Electrostatic interaction of intermediate strength associated with maximum activity

A qualitatively important role of membrane electrostatic interactions for the activity of colicin E1 (Davidson et al., 1985; Kayalar and Düzgünes, 1986; Xu et al., 1988) and colicin A (Lakey et al., 1994) has been proposed previously. In the present study, although the electrostatic component of the binding energy (ΔG_{el}) increases monotonically as the pH is lowered to 3.4, and as the surface potential of the vesicles is increased to $\psi_o \approx -35$ mV (Fig. 4), maximum activity occurs at intermediate values of pH and ψ_o; the latter is also dependent on the presence of Ca^{2+}. The tendency for activity to be correlated with intermediate values of ΔG_{el} is shown by 1) the alkaline-directed shift of the high-pH threshold for maximum activity with LUVs with a higher x_- (Fig. 1 *A*), and 2) the acidic shift of the activity maximum in the presence of Ca^{2+} (Fig. 1 *B*, Table 1). The effect of Ca^{2+} in causing a shift in the activity-pH profile can be attributed to a shift in ψ_o with 2 mM Ca^{2+} from -30 to -25 mV. The highest activities occur in the range of acidic lipid content ($x_- = 0.3$–0.5) that corresponds to the physiological level ($x_- \sim 0.3$) found in the cytoplasmic membrane of wild-type *E. coli* membranes (Shibuya, 1992).

Explanations for the association of high colicin channel activity with a ΔG_{el} of intermediate magnitude must take into account the rapid transition of the binding from an electrostatic to a predominantly nonelectrostatic mode (Fig. 3, *A* and *A*). The colicin channel polypeptide initially binds to the membrane as a soluble protein (Fig. 7 *A*), with electrostatic interactions dominating, described as Boltzmann accumulation at the membrane surface (Fig. 7 *B*). This is followed by partial unfolding and close association with the interfacial region (White and Wimley, 1994) (Fig. 7 *C*) and perhaps insertion into the hydrophobic core (Fig. 7 *D*). The latter steps largely involve nonelectrostatic (presumably hydrophobic) interactions, with some contribution from electrostatic forces. The binding of a soluble protein at low pH to a membrane is fundamentally different from the insertion of a protein such as colicin into the bilayer, essentially because the former does not exhibit the tertiary unfolding and hydrophobic insertion into the interfacial layer or bilayer demonstrated for colicin. The contrasting response to a repulsive electrostatic interaction upon a shift to high pH is therefore diagnostic of a membrane-active (Fig. 3 *A*) as opposed to a peripherally bound (Fig. 3 *C*) polypeptide.

Question of whether the colicin channel hydrophobic hairpin spontaneously inserts into the bilayer

Although a common model for the binding of the colicin E1 or Ia channel to a membrane features a transmembrane configuration for the C-terminal hydrophobic hairpin (Merrill and Cramer, 1990; Rath et al., 1991; Song et al., 1991; Zhang and Cramer, 1992; Mel and Stroud, 1993; Shin et al., 1993; Palmer and Merrill, 1994; Slatin et al., 1994), it has

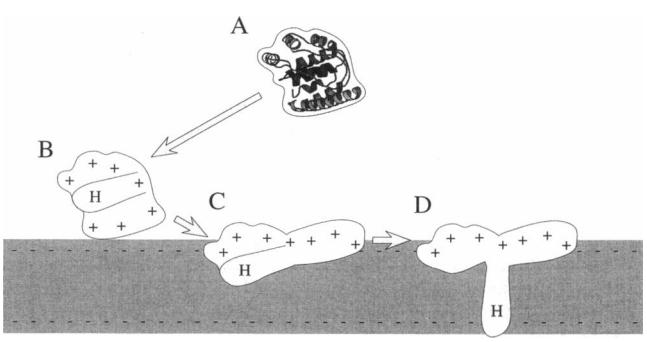

FIGURE 7 Conceptual representation of the binding of the colicin E1 channel domain to a negatively charged membrane. (*A*) Soluble structure of the colicin E1 channel polypeptide, P190 (Elkins et al., 1995), showing the sandwich structure with helices 1–2 at the bottom, helices 8–10 in the middle, and helices 3–7 at the top. (*B*) Boltzmann accumulation of the positively charged P190 at the negative membrane surface, showing an outline of the structure in *A* with the hydrophobic hairpin indicated (H ≡ helices 8–9). (*C*) Partially unfolded, membrane-bound state of P190 with helices 1–2, 10 displaced to produce the "penknife" conformation (Lakey et al., 1992). (*D*) Membrane-inserted state of P190 with the hydrophobic hairpin inserted *trans* bilayer in the "umbrella" conformation (Parker et al., 1990).

been concluded in several studies on the binding properties of the colicin A channel polypeptide (Lakey et al., 1993; Massotte et al., 1993; Jeanteur et al., 1994) that this hairpin does not insert spontaneously into the bilayer. It appears that the latter studies were carried out with membranes consisting of 100% PG lipid ($x_- = 1.0$). Considering the data of Figs. 1 *A* and 6, it is reasonable to infer that under these conditions the colicin channel did not form transmembrane segments because of the electrostatic constraints imposed by the predominant content of anionic lipid. The explanation of the disagreement over whether the hydrophobic hairpin translocates across the hydrophobic bilayer may be 1) it translocates with a physiological level of 30–40% anionic lipid, and 2) it does not with the nonphysiological 100% DOPG lipid.

We thank Drs. A. Szabo and J. Brennan for measurement of the R_0 value for fluorescence resonance energy transfer from colicin E1 to TNP-PE, S. L. Schendel for helpful discussions, and T. Wu for the preparation of colicin E1, P178, and P190.

This research was supported by National Institutes of Health grant GM-18457.

REFERENCES

Bishop, L. J., E. S. Bjes, V. L. Davidson, and W. A. Cramer. 1985. Localization of the immunity protein-reactive domain in unmodified and chemically modified COOH-terminal peptides of colicin E1. *J. Bacteriol.* 164:237–244.

Brunden, K. R., W. A. Cramer, and F. S. Cohen. 1984. Purification of a small receptor-binding peptide from the central region of the colicin E1 molecule. *J. Biol. Chem.* 259:190–196.

Bullock, J. O., F. S. Cohen, J. R. Dankert, and W. A. Cramer. 1983. Comparison of the macroscopic and single channel conductance properties of colicin E1 and its COOH-terminal tryptic peptide. *J. Biol. Chem.* 258:9908–9912.

Cleveland, M. V., S. Slatin, A. Finkelstein, and C. Levinthal. 1983. Structure-function relationships for a voltage-dependent ion channel: properties of COOH-terminal fragments of colicin E1. *Proc. Natl. Acad. Sci. USA.* 80:3706–3710.

Cramer, W. A., Y.-L. Zhang, S. Schendel, A. R. Merrill, H. Y. Song, C. V. Stauffacher, and F. S. Cohen. 1992. Dynamic properties of the colicin E1 ion channel. *FEMS Microbiol. Immunol.* 105:71–82.

Dankert, J., S. M. Hammond, and W. A. Cramer. 1980. Reversal by trypsin of the inhibition of active transport by colicin E1. *J. Bacteriol.* 143: 594–602.

Dankert, J. R., Y. Uratani, C. Grabau, W. A. Cramer, and M. Hermodson. 1982. On a domain structure of colicin E1: a COOH-terminal peptide fragment active in membrane depolarization. *J. Biol. Chem.* 257: 3857–3863.

Davidson, V. L., K. R. Brunden, and W. A. Cramer. 1985. Acidic pH requirement for insertion of colicin E1 into artificial membrane vesicles: relevance to the mechanism of action of colicins and certain toxins. *Proc. Natl. Acad. Sci. USA.* 82:1386–1390.

Elkins, P., A. Bunker, W. A. Cramer, and C. Stauffacher. 1995. The crystal structure of the channel-forming domain of colicin E1. *Biophys. J.* 68:A369.

Elkins, P., H. Y. Song, W. A. Cramer, and C. V. Stauffacher. 1994. Crystallization and characterization of colicin E1 channel-forming polypeptides. *Proteins.* 19:150–157.

Engelman, D. M., and T. A. Steitz. 1981. The spontaneous insertion of proteins into and across membranes: the helical hairpin hypothesis. *Cell.* 23:411–422.

González-Mañas, J. M., J. H. Lakey, and F. Pattus. 1993. Interaction of the colicin A pore-forming domain with negatively charged phospholipids. *Eur. J. Biochem.* 211:625–633.

Gould, J. M., and W. A. Cramer. 1977. Studies on the depolarization of the *Escherichia coli* cell membrane by colicin E1. *J. Biol. Chem.* 252: 5491–5497.

Guihard, G., H. Benedetti, M. Besnard, and L. Letellier. 1993. Phosphate efflux through the channels formed by colicins and phage T5 in *Escherichia coli* cells is responsible for the fall in cytoplasmic ATP. *J. Biol. Chem.* 268:17775–17780.

Heymann, J. B., S. D. Zakharov, Y.-L. Zhang, and W. A. Cramer. 1996. Characterization of electrostatic and non-electrostatic components of protein-membrane binding interactions. *Biochemistry.* 35:2717–2725.

Hille, J. D. R., G. M. Donné-Op den Kelder, P. Sauve, G. H. de Haas, and M. R. Egmond. 1981. Physicochemical studies on the interaction of pancreatic phospholipase A2 with a micellar substrate analog. *Biochemistry.* 20:4068–4073.

Hope, M. J., M. B. Bally, G. Webb, and P. R. Cullis. 1985. Production of large unilamellar vesicles by a rapid extrusion procedure. Characterization of size distribution, trapped volume and ability to maintain a membrane potential. *Biochim. Biophys. Acta.* 812:55–65.

Janin, J., and C. Chothia. 1978. Role of hydrophobicity in the binding of coenzymes. Appendix: translational and rotational contribution to the free energy of dissociation. *Biochemistry.* 17:2943–2948.

Jeanteur, D., F. Pattus, and P. A. Timmins. 1994. Membrane-bound form of the pore-forming domain of colicin A. *J. Mol. Biol.* 235:898–907.

Kayalar, C., and N. Düzgünes. 1986. Membrane action of colicin E1: detection by the release of carboxyfluorescein and calcein from liposomes. *Biochim. Biophys. Acta.* 860:51–56.

Kopecky, A. L., D. P. Copeland, and J. E. Lusk. 1975. Viability of *Escherichia coli* treated with colicin K. *Proc. Natl. Acad. Sci. USA.* 72:4631–4634.

Lakey, J. H., D. Duché, J.-M. González-Mañas, D. Baty, and F. Pattus. 1993. Fluorescence energy transfer distance measurements. The hydrophobic helical hairpin of colicin A in the membrane bound state. *J. Mol. Biol.* 230:1055–1067.

Lakey, J. H., J. M. González-Mañas, F. G. van der Goot, and F. Pattus. 1992. The membrane insertion of colicins. *FEBS Lett.* 307:26–29.

Lakey, J. H., M. W. Parker, J.-M. González-Mañas, D. Duché, G. Vriend, D. Baty, and F. Pattus. 1994. The role of electrostatic charge in the membrane insertion of colicin A. Calculation and mutation. *Eur. J. Biochem.* 220:155–163.

Lakowicz, J. R. 1983. Principles of Fluorescence Spectroscopy. Plenum Press, New York.

Lantzsch, G., H. Binder, and H. Heerklotz. 1994. Surface area per molecule in lipid/$C_{12}E_n$ membranes as seen by fluorescence resonance energy transfer. *J. Fluoresc.* 4:339–343.

Massotte, D., M. Yamamoto, S. Scianimanico, O. Sorokine, A. van Dorsselaer, Y. Nakatani, G. Ourisson, and F. Pattus. 1993. Structure of the membrane-bound form of the pore-forming domain of colicin A: a partial proteolysis and mass spectrometry study. *Biochemistry.* 32: 13787–13794.

McLaughlin, S. 1989. The electrostatic properties of membranes. *Annu. Rev. Biophys. Biophys. Chem.* 18:113–136.

Mel, S. F., and R. M. Stroud. 1993. Colicin Ia inserts into negatively charged membranes at low pH with a tertiary but little secondary structural change. *Biochemistry.* 32:2082–2089.

Merrill, A. R., and W. A. Cramer. 1990. Identification of a voltage-responsive segment of the potential-gated colicin E1 ion channel. *Biochemistry.* 29:8529–8534.

New, R. R. C. 1990. Characterization of liposomes. *In* Liposomes: A Practical Approach. IRL Press, Oxford.

Nozaki, Y., and C. Tanford. 1967. Examination of titration behavior. *Methods Enzymol.* 11:715–734.

Palmer, L. R., and A. R. Merrill. 1994. Mapping the membrane topology of the closed state of the colicin E1 channel. *J. Biol. Chem.* 269: 4187–4193.

Parker, M. W., F. Pattus, A. D. Tucker, and D. Tsernoglou. 1989. Structure of the membrane-pore-forming fragment of colicin A. *Nature.* 337: 93–96.

Parker, M. W., J. P. M. Postma, F. Pattus, A. D. Tucker, and D. Tsernoglou. 1992. Refined structure of the pore-forming domain of colicin A at 2.4 Å resolution. *J. Mol. Biol.* 224:639–657.

Parker, M. W., A. D. Tucker, D. Tsernoglou, and F. Pattus. 1990. Insights into membrane insertion based on studies of colicins. *Trends Biochem. Sci.* 15:126–129.

Peterson, A. A., and W. A. Cramer. 1987. Voltage-dependent, monomeric channel activity of colicin E1 in artificial membranes. *J. Membr. Biol.* 99:197–204.

Peterson, G. L. 1977. A simplification of the protein assay method of Lowry et al. which is more generally applicable. *Anal. Biochem.* 83: 346–356.

Phillips, S. K., and W. A. Cramer. 1973. Properties of the fluorescence probe response associated with the transmission mechanism of colicin E1. *Biochemistry.* 12:1170–1176.

Rath, P., O. Bousché, A. R. Merrill, W. A. Cramer, and K. J. Rothschild. 1991. Fourier transform infrared evidence for a predominantly α-helical structure of the membrane bound channel forming COOH-terminal peptide of colicin E1. *Biophys. J.* 59:516–522.

Schendel, S. L., and W. A. Cramer. 1994. On the nature of the unfolded intermediate in the in vitro transition of the colicin E1 channel domain from the aqueous to the membrane phase. *Protein Sci.* 3:2272–2279.

Seelig, J., S. Nebel, P. Ganz, and C. Bruns. 1993. Electrostatic and non-polar peptide-membrane interactions. Lipid binding and functional properties of somatostatin analogues of charge z = +1 to z = +3. *Biochemistry.* 32:9714–9721.

Shibuya, I. 1992. Metabolic regulations and biological functions of phospholipids in *Escherichia coli. Prog. Lipid Res.* 31:245–299.

Shin, Y.-K., C. Levinthal, F. Levinthal, and W. L. Hubbell. 1993. Colicin E1 binding to membranes: time-resolved studies of spin-labeled mutants. *Science.* 259:960–963.

Slatin, S. L., X.-Q. Qiu, K. S. Jakes, and A. Finkelstein. 1994. Identification of a translocated protein segment in a voltage-dependent channel. *Nature.* 371:158–161.

Song, H. Y., F. S. Cohen, and W. A. Cramer. 1991. Membrane topography of ColE1 gene products: the hydrophobic anchor of the colicin E1 channel is a helical hairpin. *J. Bacteriol.* 173:2927–2934.

van der Goot, F. G., N. Didat, F. Pattus, W. Dowhan, and L. Letellier. 1993. Role of acidic lipids in the translocation and channel activity of colicins A and N in *Escherichia coli* cells. *Eur. J. Biochem.* 213: 217–221.

Webster, R. E. 1991. The *tol* gene products and the import of macromolecules into *Escherichia coli. Mol. Microbiol.* 5:1005–1011.

White, S. H., and W. C. Wimley. 1994. Peptides in lipid bilayers: structural and thermodynamic basis for partitioning and folding. *Curr. Opin. Struct. Biol.* 4:79–86.

Xu, S., W. A. Cramer, A. A. Peterson, M. Hermodson, and C. Montecucco. 1988. Dynamic properties of membrane proteins: reversible insertion into membrane vesicles of a colicin E1 channel-forming peptide. *Proc. Natl. Acad. Sci. USA.* 85:7531–7535.

Zakharov, S. D., J. B. Heymann, Y.-L. Zhang, and W. A. Cramer. 1995. Colicin E1 ion channel binding with membranes: an optimum electrostatic interaction is required for activity. *Biophys. J.* 68:A368.

Zhang, Y.-L., and W. A. Cramer. 1992. Constraints imposed by protease accessibility on the trans-membrane and surface topography of the colicin E1 channel. *Protein Sci.* 1:1666–1676.

Zhang, Y.-L., J. B. Heymann, and W. A. Cramer. 1994. Characterization of electrostatic interactions in the initial binding of the colicin E1 channel domain to membrane vesicles. *Biophys. J.* 66:A439.