Protein Science (1994), *3*:2272–2279. Cambridge University Press. Printed in the USA.

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

SHARON L. SCHENDEL AND WILLIAM A. CRAMER
Department of Biological Sciences, Purdue University, West Lafayette, Indiana 47907

(RECEIVED September 1, 1994; ACCEPTED October 10, 1994)

Abstract

The transition of the colicin E1 channel polypeptide from a water-soluble to membrane-bound state occurs in vitro at acid pH values that are associated with an unfolded channel structure whose properties qualitatively resemble those of a "molten globule," or "compact unfolded," intermediate state. The role of such a state for activity was tested by comparing the pH dependence of channel-induced solute efflux and the amplitude of the near-UV CD spectrum. The requirement of a partly unfolded state for activity was shown by the coincidence of the onset of channel activity measured for 4 different lipid compositions with the decrease in near-UV CD amplitude as a function of pH. Tertiary constraints on the 3 tryptophans of the colicin channel, assayed by the amplitude of the near-UV CD spectrum, are retained over the pH range 3–4 where channel activity could be measured and, as well, at pH 2. In addition, the tryptophan fluorescence emission spectrum is virtually unchanged over the pH range 2–6. The temperature independence of the near-UV spectrum at pH 3–6 up to 70 °C implies that the colicin E1 channel polypeptide is more stable than that of colicin A. A transition between 53 and 58 °C in the amplitude of the near-UV CD is consistent with preservation of part of the hydrophobic core in a destabilized state at pH 2. Thus, the unfolded state associated with colicin activity at acidic pH has the properties of a "compact unfolded" state, having some, but not all of the properties of a "molten globule."

The small effect on local membrane acidity of a physiological acidic membrane lipid content, the retention of significant near-UV CD amplitude down to pH 2, and the small extent of immersion of the 40-Å globular colicin channel polypeptide in the 10-Å lower pH layer at the membrane surface make it unlikely that a local lower pH at the membrane surface significantly facilitates formation of an unfolded intermediate.

Keywords: membrane protein import; translocation

The transition from the water-soluble to a membrane-inserted state of toxins, such as diphtheria (London, 1992) and *Pseudomonas* exotoxin A (Jiang & London, 1990), toxin-like molecules such as the pore-forming colicins (Cramer et al., 1990; Lakey et al., 1994), and the human complement glycoprotein, C9 (Lohner & Esser, 1991), involve large structural changes. The nature of the structural intermediates involved in these changes is of interest in the context of the problems of protein folding and of protein insertion into membranes. It has been proposed that structure changes associated with protein unfolding that are relevant to the insertion process are induced by: (1) the bulk phase acidic pH required for activity of toxins such as diphtheria (London, 1992) and the channel-forming colicins (Davidson et al., 1985; Merrill et al., 1990); and (2) the more acidic environment near the negatively charged membrane surface (van der Goot et al., 1991; Muga et al., 1993).

Diphtheria toxin and the colicin E1 channel domain share the common features in response to acidic bulk phase pH of (1) a hydrophobic character inferred by their partition from water into a nonionic detergent phase, and (2) increased accessibility to probes such as protease and, in the case of the colicin E1 channel, acrylamide (Merrill et al., 1990). (3) It has been proposed that the acidic active state of diphtheria toxin is characterized by a partial (Zhao & London, 1986) or massive (Ramsay et al., 1989) unfolding of the protein. In the partially unfolded state, it was reported that tertiary but not secondary structure is disrupted, corresponding to the behavior of proteins in the "molten globule" conformation (London, 1992). The loss of tertiary structure of the colicin A channel polypeptide at pH val-

Reprint requests to: William A. Cramer, Department of Biological Sciences, Purdue University, West Lafayette, Indiana 47907; e-mail: wac@bilbo.bio.purdue.edu.

Abbreviations: ANS, 1-anilino-naphthalene-8-sulfonate; DOPC, dioleoyl phosphatidylcholine; DOPG, dioleoyl phosphatidylglycerol; GdnHCl, guanidine hydrochloride; LUV, SUV, large and small unilamellar vesicles; PG, phosphatidylglycerol.

ues <2.5 assayed by loss of the near-UV CD spectrum, implied that a "molten globule" intermediate of the colicin A channel might be an intermediate in its insertion into 100% anionic (DOPG) membranes (van der Goot, 1991; Muga et al., 1993). The "molten globule" intermediate in protein folding is characterized by: (1) conservation of the native secondary structure; (2) loss of tertiary structure or poorly defined tertiary structure contacts; (3) a volume approximately unchanged from that of the native protein; and (4) an increased accessibility of the hydrophobic core to hydrophobic dyes such as ANS (Haynie & Freire, 1993; Skolnick et al., 1993). The protease-, acrylamide-accessible low pH (pH 3.5) translocation-competent state of the colicin E1 channel domain was shown to possess an approximately unchanged effective hydrodynamic radius (Merrill et al., 1990), which, together with an increased hydrophobic character, was attributed to an unfolded or dynamic state. The above properties of the colicin E1 channel have also been described as "molten globule" (Ptitsyn, 1992). The purpose of the present study is to further examine the requirement of an in vitro acid-unfolded state of the colicin channel for activity, to further examine the nature of this state, and to inquire whether this state can be characterized as "molten-globule."

Results

Far-UV spectra of P190

The loss of the near-UV but not far-UV CD spectrum of the colicin A channel at pH values <2.5 implied loss of tertiary but not secondary structure, and the role of a "molten globule" state as an intermediate in the colicin insertion into DOPG vesicles (van der Goot et al., 1991). The far-UV (200–250 nm) CD spectrum of the 190-residue colicin E1 C-terminal channel polypeptide (P190) is dominated by the amide bond absorption, is sensitive to the presence of ordered secondary structure, and shows the presence of 50–60% α-helical secondary structure (Fig. 1A), as shown previously (Brunden et al., 1984). Little change was seen in the shape of the far-UV spectrum from pH 2 to 6, indicating that the α-helical nature of the P190 channel polypeptide remains intact over this pH range, as found previously for the colicin A channel polypeptide (van der Goot et al., 1991). The amplitude of the P190 far-UV spectrum can be almost totally eliminated by incubation with concentrations of GdnHCl greater than 3 M (data not shown).

Near-UV spectra of P190

Information regarding the tertiary structure can be obtained from the near-UV CD spectrum. The near-UV CD spectrum of P190 exhibits 2 intense bands of positive ellipticity at 284 and 292 nm (Fig. 1B). The position and intensity of these bands indicate that one or more of the 3 tryptophans (Trp 424, Trp 460, and Trp 495) of the P190 polypeptide is in a constrained environment. The P190 polypeptide has 9 tyrosines and 8 phenylalanines, 3- and 2-fold greater, respectively, than the content of the colicin A channel polypeptide (Parker et al., 1992). However, in contrast to the near-UV CD spectrum of the native colicin A channel polypeptide (van der Goot et al., 1991; Muga et al., 1993), which has negative ellipticity in near-UV CD, no significant ellipticity bands were observed in the region diagnostic of tyrosine (275–282 nm) or phenylalanine (255–270 nm). This suggests (1) that these Tyr and Phe residues, particularly the Tyr, with a higher extinction coefficient relative to phenylalanine, are unconstrained; (2) because both positive or negative ellipticity can be observed for tyrosine and phenylalanine, there may be a cancellation effect arising from the different environments of these residues; and (3) a fraction of the ellipticity arising from the tyrosine may be dominated by overlapping tryptophan intensity.

The amplitude of the near-UV CD spectrum of P190 at 293 nm shows a decrease of 25% and 35–40% at pH 3 and 2, respectively, compared to pH 6 (Fig. 1B). However, the structure and shape of the spectrum was retained across the same pH range (Fig. 1B). This is in contrast to that seen for colicin A, which shows a complete loss of near-UV ellipticity and spectral characteristics at pH 2. Cytochrome *c* (Ohgushi & Wada, 1983), human carbonic anhydrase B (Jagannadham & Balasubramanian, 1985), and α-lactalbumin (Dolgikh et al., 1981) are other examples of proteins that experience a nearly complete loss of the near-UV spectrum at pH 2. This implies that, in the case of the colicin E1 P190 polypeptide, a large degree of the tertiary structure is maintained, the environment and mobility of the tryptophan residues is not greatly altered at pH 3, and these tryptophan residues remain significantly restrained at pH 2. The amplitude of the P190 near-UV spectrum is slightly affected by 1 M GdnHCl (Fig. 1C, curve b) and is almost totally eliminated if it is incubated in GdnHCl-HCl at concentrations greater than 3 M (Fig. 1C, curve d), showing that by this criterion the pH 2 state (Fig. 1C, curve c) is far from denatured.

A "melting curve" of the channel polypeptide was determined through the temperature dependence of the near-UV CD amplitude (Fig. 1D). The near-UV CD spectrum of the colicin A channel polypeptide at pH 5 completely collapses by 70 °C with a midpoint of 65 °C (Muga et al., 1993). In contrast, the amplitude and shape of the near-UV CD spectrum of the colicin E1 channel at pH 6 and 3 is independent of temperature up to 70 °C (Fig. 1D, curves a, b). At 75 °C, the polypeptide precipitates. Thus, the colicin E1 channel polypeptide appears more stable than colicin A. The E1 channel polypeptide at pH 2 shows a unique melting transition at 53–58 °C (Fig. 1D, curve c), indicating that part of the hydrophobic core is preserved at this pH.

It has been proposed that the complete loss of spectral amplitude of the near-UV CD spectrum of the colicin A channel polypeptide in solution at pH 2–2.5 is reporting a total loss of tertiary structure contact that also occurs at the membrane surface when the colicin channel polypeptide is bound. The vesicles used in those experiments consisted of 100% anionic lipid, for which the surface pH is 1.6 pH units more acid than the bulk (van der Goot et al., 1991). On the other hand, the physiological content of anionic lipid in the *Escherichia coli* cytoplasmic membrane is approximately 30% (Shibuya, 1992). The pH dependence of channel activity for DOPG-DOPC liposomes containing 10–40% anionic lipid is shown in Figure 2A (10% [function b], 20% [function c]) and 2B (30% [function b], 40% [function c]), where the activity functions are also compared with the pH dependence of the amplitude of the near-UV CD measured at 293 nm (function a). Taking into account Guoy-Chapman theory (McLaughlin, 1989) applied to the medium consisting of 0.1 M ionic strength and 2 mM Ca^{2+} (van Dijck et al., 1978; Lau et al., 1981), the difference between the pH at the membrane surface and in the bulk is negligible or small,

0–0.3 pH units, for these liposomes. Under these conditions, with similar values of bulk and surface pH, the pH dependence of relative activity measured for these liposomes should be similar. Furthermore, the bulk phase pH dependence of the amplitude of the near-UV CD spectrum of the channel polypeptide should be the same or similar to that which would be measured at the membrane surface.

The data of Figure 2 show: (1) the pH dependence of the activity is similar for anionic lipid contents of 10–40%, although the absolute level of the channel activity increases as the anionic lipid content of the vesicles is increased from 10–20% to 30–40%; (2) the onset of activity coincides with the decrease of near-UV CD amplitude at a pH of approximately 4.0; and (3) at pH 3.0, in 40% DOPG/DOPC vesicles, where the activity is $\sim 1.5 \times 10^4$ ions/channel-s (averaged over a time interval of approximately 30 s), the amplitude of the near-UV CD has decreased by approximately 25%. This implies that the onset of colicin E1 channel activity in vitro is accompanied by a partial, but far from complete, loss of tertiary structure in solution or at the membrane surface. (4) Even at pH 2, which is 1 unit below the minimum pH at which activity is measured in the present work, and 1.5–2.0 units below that at which it has been measured previously (Peterson & Cramer, 1987; Merrill et al., 1990), the amplitude of the near-UV CD is 3/5–2/3 that at pH 6 (Figs. 1B, 2A,B).

In the lower pH region of the activity measurements shown in Figure 2A and B, the ionic state of the PG lipid could affect the surface potential. However, titrations of the pK_a of the PG lipid in DOPG:DOPC (30:70, w/w) vesicles in the presence of 2 mM Ca^{2+} defined the pK_a of the PG lipid to be 2.3–2.4 (data not shown). The pK value of dilauryl-PG in the absence of Ca^{2+} was reported to be 3.1 (van Dijck et al., 1978). Thus, al-

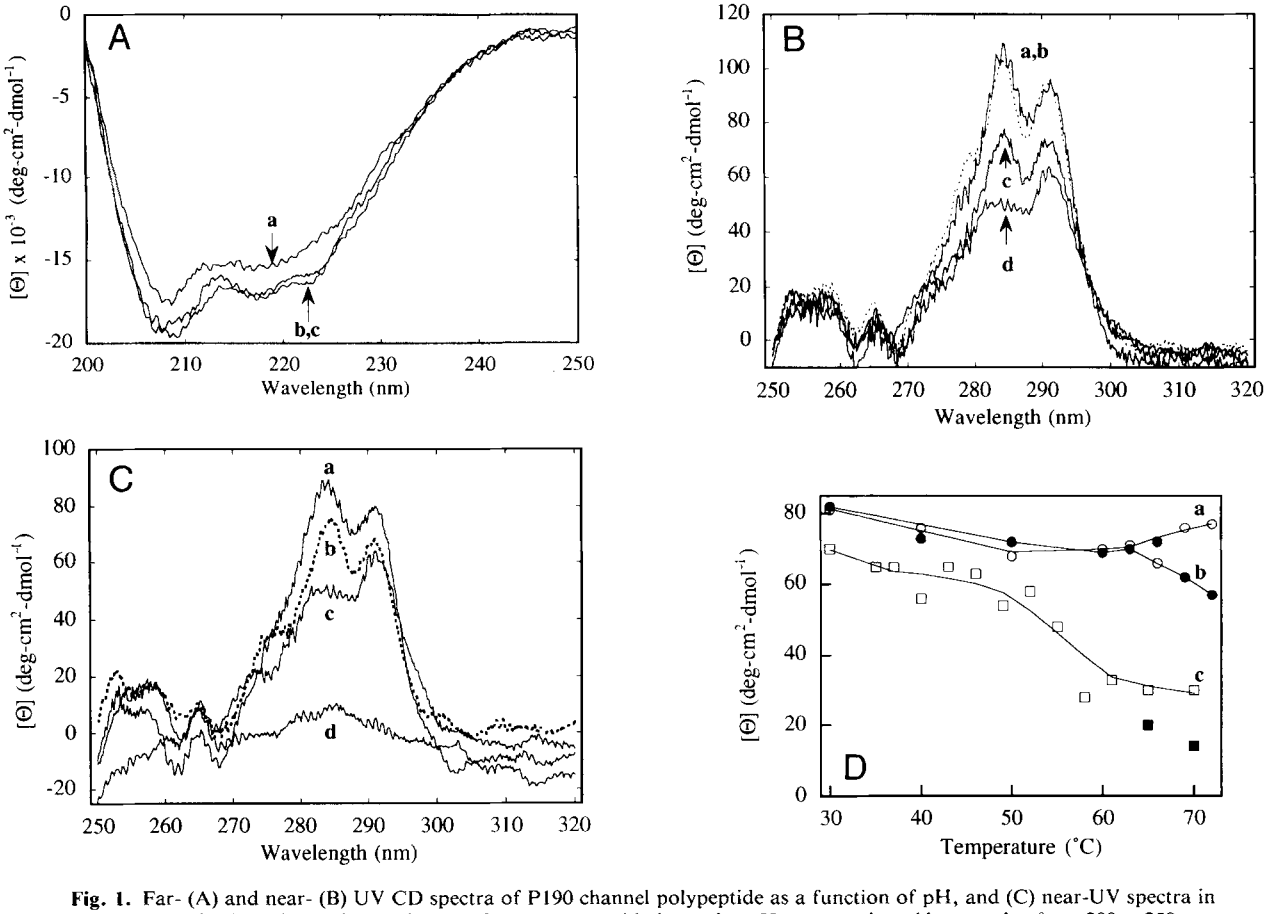


Fig. 1. Far- (A) and near- (B) UV CD spectra of P190 channel polypeptide as a function of pH, and (C) near-UV spectra in the presence of GdnHCl. **A:** Changes in secondary structure with decreasing pH were monitored by scanning from 200 to 250 nm. P190 (0.05 mg/mL in 0.1-cm-pathlength cell, thermostatted at 25 °C), in 30 mM Na-phosphate, pH 6 (curve a), or 30 mM glycine-HCl (pH 3 and 2, curves b and c, respectively). **B:** Near-UV spectra were measured as a function of pH from 250 to 320 nm. P190 (0.5 mg/mL), in either 30 mM Na-phosphate, pH 6 (curve a), 30 mm sodium acetate, pH 4 (curve b, dashed line), or 30 mM glycine-HCl for pH 3 and 2 (curves c and d, respectively); 1-cm-pathlength cell. **C:** Near-UV spectra of P190 in GdnHCl. P190 (0.5 mg/mL) in 30 mM Na-phosphate (pH 6) containing either 0 M (curve a), 1 M (curve b), or 6 M (curve d) GdnHCl incubated for at least 30 min before measurement. The spectrum of P190 in 30 mM glycine-HCl, pH 2 (curve c), is shown for comparison. **D:** Temperature dependence of the near-UV ellipticity at 293 nm of P190 as a function of pH. Near-UV spectra of P190 (0.5 mg/mL) at increasing temperatures was measured at pH 6 (curve a, open circles, solid line), pH 3 (curve b, filled circles), and pH 2 (curve c, open squares; data points at 65 and 70 °C have been corrected to reflect the similar decrease in intensity above 65 °C seen at pH 3; actual data at 65 and 70 °C, closed squares). All CD spectra represent the average of 4 scans corrected for background intensity by subtraction of the appropriate buffer blank. Bandwidth, 1 nm; full-scale sensitivity, 10 mdeg; time constant, 2 s; cuvette thermostatted at 25 °C.

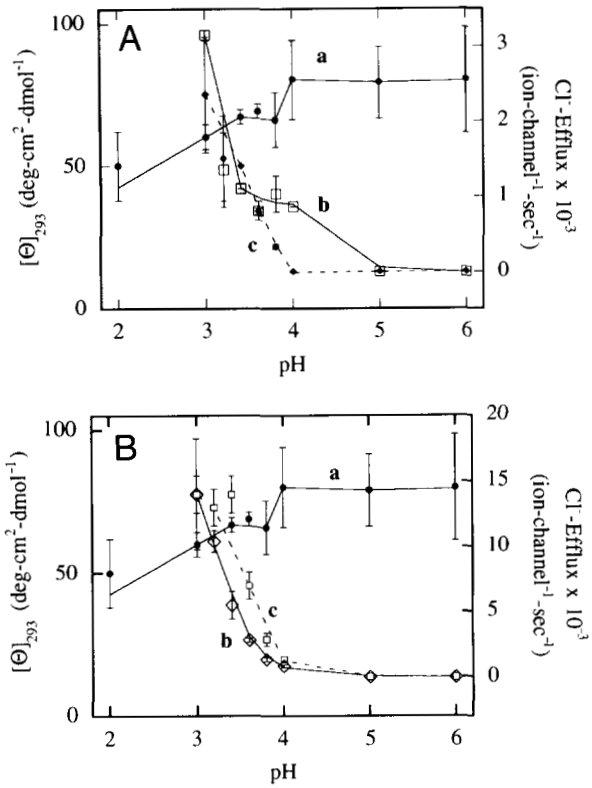


Fig. 2. Comparison of near-UV CD amplitude of P190 and P190 channel activity as a function of pH. **A:** For activity measurements, membrane vesicles composed of either 10% DOPG/90% DOPC (curve b) or 20% DOPG/80% DOPC (mol/mol) (curve c) were added at a concentration of 0.05 mg/mL to DMG buffer, and an inside-negative diffusion potential was induced by the addition of valinomycin. P190 was subsequently added to a concentration of either 24 ng/mL or 48 ng/mL. Cl^- efflux induced by addition of P190 was measured as described in the Materials and methods. **B:** Procedure as in A with LUV composed of either 30% DOPG/70% DOPC (curve b) or 40% DOPG/60% DOPC (curve c) and a final P190 concentration of 12 ng/mL. For CD, P190 was dissolved in either 30 mM Na_2HPO_4 buffer titrated to the given pH with phosphoric acid, 30 mM glycine-HCl (pH 2–3), 30 mM sodium acetate (pH 4–5), or 30 mM sodium phosphate (pH 6). All near-UV measurements were made using a 1-cm-pathlength cell and a P190 concentration of 0.5 mg/mL. The magnitude of the mean residue ellipticity at 293 nm as a function of pH is plotted as curve a in both A and B.

though the surface charge of the membrane vesicles is decreased at pH 3.0, the magnitude of the effect and its resultant influence on the activity is estimated to be only approximately 20%.

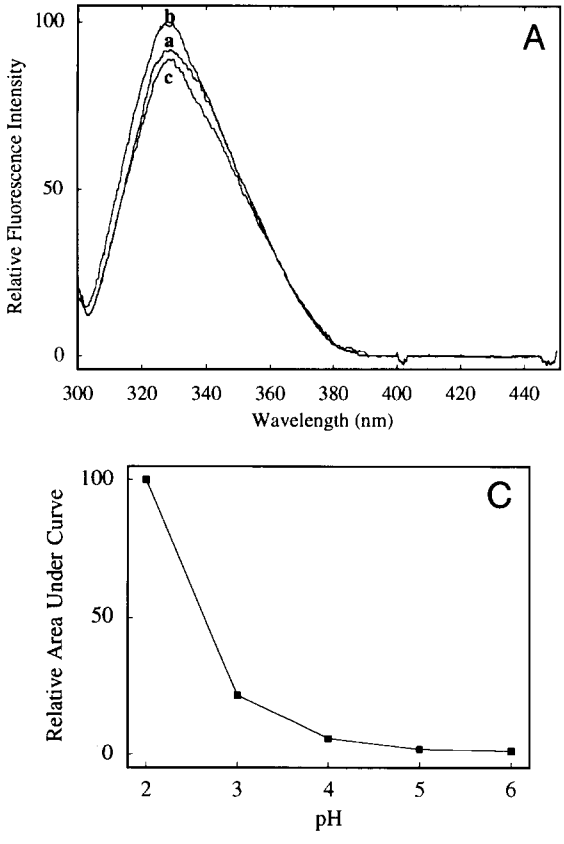
The intrinsic fluorescence emission spectra of the 3 tryptophans in the channel polypeptide, Trp 424, 460, and 495, provide an additional indication of its unfolded state at acidic pH. These spectra imply that the environment of the 3 Trp is essentially unchanged at pH 2 and 3 (Fig. 3A, curves c, b, emission maxima 327, 329 nm) relative to pH 6 (Fig. 3A, curve a, emission maximum 328 nm), with very similar emission maxima and intensities.

The fluorescence intensity and emission maximum of the polarity-dependent probe ANS is another indicator of the hydrophobic character of a protein (Stryer, 1965). The increased hydrophobic character of proteins in the acid pH range has been indicated by 50–100-fold increases of ANS fluorescence intensity for bovine carbonic anhydrase and α-lactalbumin (Semisotnov et al., 1991). The increased hydrophobicity of the colicin E1 channel polypeptide at acidic pH (Merrill et al., 1990) has been shown by its increased partition into nonionic detergent. The acidic pH dependence of the increase in hydrophobicity of the colicin E1 channel sensed by the ANS probe is shown in Figure 3B and C. The ANS emission maximum in the presence of the channel decreases from 491 nm at pH 6 to 483, 479, and 479 nm at pH 4, 3, and 2, respectively, with the largest shift in the emission peak occurring between pH 6 and 4. The relative fluorescence intensities at pH 6, 4, and 3, measured by the integrated area under the spectrum are 1.0, 5.9, and 22, normalized to 100 at pH 2. It is possible that part of the ANS response to decreasing pH reflects the electrostatic effect of neutralizing the protein carboxylates below pH 3. Nevertheless, the response from pH 6 to 3 is similar to the pH profile of partition of the channel polypeptide into nonionic detergent (Merrill et al., 1990).

Discussion

In the acid pH range where the colicin E1 and A channel polypeptides have been shown to be active in vitro, several properties, increased hydrophobicity, increased accessibility to protease and acrylamide probes, and the approximately constant size relative to the neutral pH state, imply a more mobile and dynamic molecule that is partly unfolded (Merrill et al., 1990). Some questions that arise are: (1) Does the formation of this unfolded state correlate well with the onset of activity? (2) Regarding the nature of the unfolded state, is it "molten globule" as defined by Ptitsyn (1992) and inferred by van der Goot et al. (1991) and Muga et al. (1993), or is it better described as a more general "compact unfolded or denatured state" (Kim & Baldwin, 1990)? It should be noted that one difference of perspective in considering such partly denatured states of the colicin and toxin molecules is that, in contrast to other enzyme systems where they have been studied, in this case they are considered to be necessary for activity. (3) Is the propensity of the colicin channel polypeptide to form an unfolded state enhanced by the especially low pH at the membrane surface toward which it expresses activity?

Regarding question (1), it is clear from the comparison of the pH dependence of activity and near-UV CD spectral amplitude that in vitro activity is associated with an unfolded intermediate of some kind. For question (2), it was inferred that the loss of the near-UV CD spectrum below pH 2.5 for the colicin A channel polypeptide implied that the molecule could assume a "molten globule" state at the surface of 100% anionic DOPG membranes, where the pH would be approximately 1.6 pH units more acidic than that in bulk solution. The possibility of a "molten globule" intermediate functioning in translocation of membrane proteins has been proposed (Bychkova et al., 1988). The problems with the existence of such a "molten globule" intermediate for the colicin channel polypeptide are: (a) It would not provide for a role of this intermediate with synthetic membranes containing a physiological level (ca. 30% for *E. coli* membranes; Shibuya, 1992) of anionic lipid. For 30% anionic lipid content, the pH at the surface of the membrane in the absence of Ca^{2+}, calculated from Guoy-Chapman theory (McLaughlin, 1989), is acid-shifted by only approximately 0.6–0.7 units. (b) It was also found that the colicin E1 channel polypeptide is relatively inactive toward liposomes containing 100% anionic (DOPG) lipid (Zakharov et al., 1995); no data on ion translocation activity in such liposomes are apparently available for the colicin A chan-



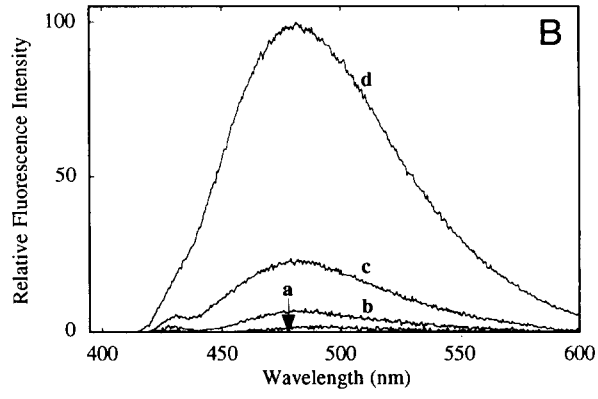


Fig. 3. A: pH dependence of P190 tryptophan fluorescence. P190 (5 μg/mL) in 30 mM Na-phosphate (pH 6, curve a), and 30 mM glycine-HCl (pH 2 and 3, curves c and b, respectively). Spectra were measured using an excitation wavelength of 293 nm. Each spectrum has been corrected for background intensity by subtraction of the appropriate buffer blank. Spectra were also recorded for pH values of 4 and 5 (data not shown), and these spectra very closely resembled those at pH 6, 3, and 2 with no significant change in emission intensity or peak wavelength. **B:** Fluorescence emission spectra of ANS-P190 polypeptide as a function of pH. P190 (5 μg/mL) with 10 μM ANS in 30 mM Na-phosphate (pH 6, curve a), 30 mM sodium acetate (pH 4, curve b), and 30 mM glycine-HCl (pH 3 and 2, curves c and d, respectively). The fluorescence emission spectra were recorded using an excitation wavelength of 372 nm and a scanning increment of 0.5 nm. Each spectrum is corrected for background response by subtraction of the spectrum of the appropriate buffer with ANS in the absence of P190. **C:** Graph of integrated intensities of ANS emission spectra as a function of pH.

nel. Concerning question (3), the contact made with the acid pH environment by the globular protein as it approaches the membrane surface is much less complete than for the totally immersed protein in bulk solution where the existence of a molten globule state has been documented at acidic (ca. pH 2) pH (Dolgikh et al., 1981; Ohgushi & Wada, 1983; Jagannadham & Balasubramanian, 1985). The Debye length for the low pH surface layer is approximately 10 Å compared to a diameter of the colicin E1 channel polypeptide of approximately 42 Å (Fig. 4), according to its hydrodynamic Stokes radius (Merrill et al., 1990). Therefore, it can be calculated that only 18% of the volume of the protein encounters a pH that has an acidity approximately equal to that at the surface. The ability of such a localized acid pH environment, in which just the "tip" of the protein is inserted into the acid bath, to cause a transition to the molten globule state has no precedent in studies on soluble proteins. A comment of this nature was made previously (Pain, 1991).

The data differ from those obtained with the colicin A channel polypeptide with respect to the major point that the amplitude of the near-UV CD spectrum, an indicator of protein tertiary structure, is mostly retained at acidic pH. At pH 3, which is the lowest pH at which activities were measured because of the interfering effect of lipid neutralization, this amplitude is 70–80% of that at neutral pH, and the channel-induced solute efflux activity with 40% anionic lipid is 1.5×10^4 Cl^-/channel-s. This is a relatively large activity for this kind of measurement (Peterson & Cramer, 1987; Merrill et al., 1990). However, it is possible from the shape of the pH profile of the activity (Fig. 2B) that it could be approximately twice as large and maximal at pH 2 if the measurement could be made. Even at pH 2, however, the amplitude of the near-UV CD spectrum of the channel polypeptide is 3/5–2/3 of that at neutral pH and the spectral shape is largely retained (Fig. 1B). Treatment of the channel polypeptide with 4–6 M GdnHCl causes loss of most of the near-UV CD spectrum (Fig. 1C). The lack of change of the environment of the tryptophans is emphasized by the similarity of the Trp emission spectra over the pH range 2–6 (Fig. 3A). These spectra indicate that the polarity of the Trp environment is the same as the native protein and very different from that of the polypeptide denatured in GdnHCl.

It is concluded that the specifically defined "molten globule" intermediate does not function in the binding-insertion of the colicin E1 channel polypeptide. It has been argued on more general considerations of protein folding that such a mobile intermediate is unlikely to function in membrane translocation of proteins constrained on the membrane surface (Haynie & Freire, 1993).

Consequences for in vivo mechanism

Nevertheless, by criteria of probe accessibility demonstrated previously (Merrill et al., 1990), increased overall hydrophobicity demonstrated in that previous study and the present one, and the incomplete but significant decrease in the near-UV CD spectrum at pH 2–3 shown here, the channel polypeptide does have a partly unfolded conformation at acidic bulk phase pH (<4.0) that is necessary for activity. It would be of interest to define

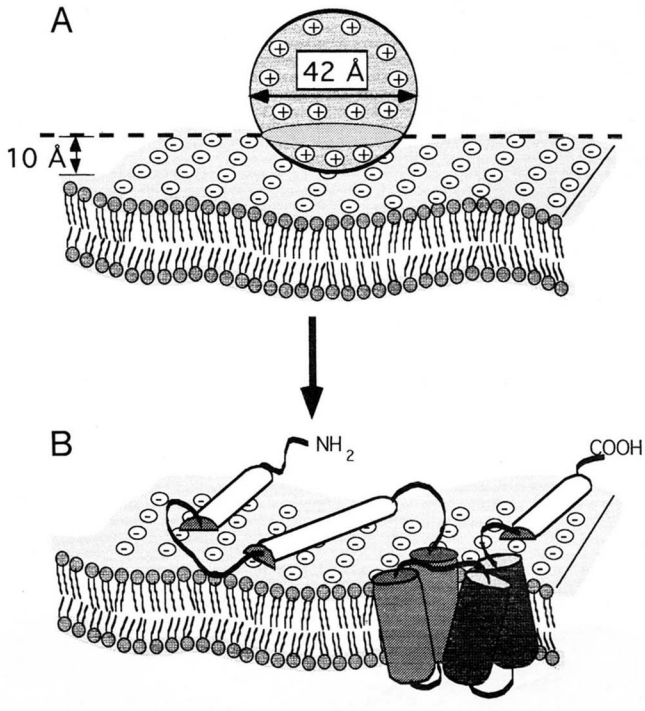


Fig. 4. Approach, binding, and initial insertion of P190 channel polypeptide to a negatively charged membrane surface. **A:** Schematic representation of a DOPC/DOPG bilayer with a net negative charge. P190 is depicted as a sphere with a diameter of 42 Å, based on its Stokes radius at pH 3.5 (Merrill et al., 1990), approaching the negatively charged membrane surface. Dashed line represents the position of the Debye length, 10 Å from the surface of the membrane. **B:** Bound intermediate in the absence of a membrane potential "umbrella" structure with inserted hydrophobic anchor hairpin (umbrella handle) and amphiphilic and charged helical segments (umbrella cover) bound to the membrane surface or interfacial layer (Zhang & Cramer, 1992).

the structural properties of this state. It appears that the state can also be created by low concentrations (~0.001%) of SDS at neutral pH (Merrill et al., 1990). One can infer from these studies that one of the roles of the in vivo intra- and intermembrane Tol protein (Webster, 1991) system in translocating the colicin from the receptor in the outer membrane to the cytoplasmic membrane is to impose a similar unfolded state on the C-terminal channel domain of the colicin.

Additional forces causing unfolding at the membrane surface

It is necessary to describe the nature of additional forces exerted on the globular protein that may contribute to its unfolding (Fig. 4A) as it encounters the surface of the membrane. These forces cause the transition to the surface-bound unfolded, partly inserted state (Fig. 4B) that has been described in studies on the binding to membrane vesicles with a physiological content of anionic lipid (Zhang & Cramer, 1992; Zakharov et al., 1995). The dependence of parameters that describe the binding, K_d, and number of lipids/channel on ionic strength, pH, and anionic lipid content imply that the initial interaction is dominated by electrostatic interactions (Zakharov et al., 1995), as for cytochrome *c* (Muga et al., 1991). This electrostatic interaction could be involved in primary binding. Alternatively, it could cause the concentration of the positively charged channel polypeptide at the membrane surface in response to the surface potential, thus allowing a primary hydrophobic interaction to be expressed (Shin et al., 1993). However, very few hydrophobic residues, only Pro 168, Ala 169, and Ile 170, in the apex of the hydrophobic hairpin helices VIII and IX) of the colicin A channel at pH 4.4 are solvent accessible (Parker et al., 1992). Of these, only Ile 170 is hydrophobic. In the adjacent residues, Leu 165 and Leu 167 are not solvent exposed and, moreover, their side chains point away from the solvent. As implied by the interhelix disulfide cross-linking study on the colicin A channel of Duché et al. (1994), helix I or helices I–II of the channel must be stripped away from the hydrophobic core helices VIII–IX in order for the channel to be able to bind to the membrane surface and for the hydrophobic hairpin to interact sufficiently with the interfacial layer of the membrane so that it can insert. It is proposed, following the suggestions of Parker et al. (1989) and Muga et al. (1993) for the mechanism of membrane interaction of the colicin A channel, that electrostatic interactions between certain basic residues (e.g., Lys 362, Lys 402, Lys 403) from the total of 24 (23 Lys, 1 Arg) are residues in P190 involved in docking to the negatively charged membrane surface. This electrostatic interaction would direct the initial contact with the membrane and the opening of the colicin channel "umbrella" structure on the membrane surface.

Materials and methods

Materials

GdnHCl (Sequanal grade) was purchased from Pierce. ANS and synthetic lipids were purchased from Molecular Probes (Eugene, Oregon) and Avanti Polar Lipids, respectively.

Purification of colicin E1 COOH-terminal polypeptide ("P190")

The 190-residue (MW = 21,046) P190 C-terminal channel polypeptide, whose construction is described in Elkins et al. (1994) was purified according to Zhang and Cramer (1992).

CD spectroscopy

CD measurements were carried out on a JASCO-J600 spectropolarimeter equipped with a temperature control accessory calibrated with *d*-10-camphor sulfonate. Measurements were taken using cells of 0.1 mm and 10 mm for far- and near-UV spectra, respectively. All spectra were recorded in 0.2-nm wavelength increments with a 2-s time constant and a full-scale sensitivity of 10 mdeg. Each spectrum is the average of 4 scans corrected for background solvent effects by subtraction of the appropriate blank. The P190 concentration was determined from its absorbance at 280 nm (extinction coefficient, $\epsilon_{mM} = 1.45$ mg^{-1}-mL) and was diluted to a concentration of 0.05 or 0.5 mg/mL for far- and near-UV measurements, respectively. P190 was dissolved in either 30 mM Na_2HPO_4 (pH 2–6) and titrated with phosphoric acid (all pH values), 30 mM glycine-HCl (pH 2–3), 30 mM sodium acetate (pH 4–5), or 30 mM Na-phosphate (pH 6). To monitor changes in secondary structure, spectra were scanned in the far-UV from 250 to 200 nm. Changes in the near UV, diagnostic of tertiary structure changes, were followed by scan-

ning from 250 to 320 nm. A value of 110 for the mean residue molecular weight was used in the calculation of the mean residue ellipticity (θ).

For experiments involving GdnHCl denaturation, P190 was dissolved at either 0.5 or 0.05 mg/mL for near and far-UV CD measurements, respectively, in 30 mM Na-phosphate, pH 6, in the presence of GdnHCl at concentrations of 0–6 M and allowed to equilibrate for 30 min prior to measurement.

Cl^--efflux measurements

LUV composed of either 10, 20, 30, or 40% DOPG and 90, 80, 70, or 60% DOPC were prepared in 10 mM KCl, 10 mM dimethylglutaric acid (DMG), 1 mM $CaCl_2$ (pH 5.0), according to Peterson and Cramer (1987). The vesicles were then diluted 200-fold and resuspended at a concentration of 0.05 mg/mL in 10 mM DMG, 100 choline nitrate (Peterson & Cramer, 1987), and 2 mM $CaNO_3$ titrated to the appropriate pH with NaOH. Valinomycin was added prior to the addition of P190 to generate a Nernst diffusion potential of 135 mV, negative inside. P190 was then added to a concentration (12–48 ng/mL) sufficient to induce release of 40–80% of the total encapsulated Cl^-, the remainder of which was released after the addition of Triton X-100 (final concentration, 0.1%). P190-induced Cl^- efflux was measured with a Cl^--specific electrode (Orion 94-17B) and a double junction reference calomel electrode (Orion 90-02) (Peterson & Cramer, 1987).

pH titration of DOPG–DOPC vesicles

LUV (30%/70% DOPG:DOPC) were sedimented by ultracentrifugation at 40,000 rpm (1 h) in a Ti-60 rotor (Beckman) and resuspended in 2 mL of the same medium as used for preparation, diluted 500-fold. Small aliquots (1–4 μL) of 0.1 M HCl were then successively added to the suspension, and the pH was recorded after equilibration.

Tryptophan fluorescence

Tryptophan fluorescence emission spectra were measured with an Aminco SLM-8000C spectrofluorimeter operated in a ratio mode with the cuvette holder thermostatted at 25 °C using an excitation wavelength of 293 nm to minimize the tyrosine contribution of tyrosine fluorescence. Emission spectra were scanned from 300 to 450 nm in 0.5-nm increments using excitation and emission slit widths of 4 nm, with an integration time of 0.5 s. The background intensity without protein was subtracted. In order to monitor the pH dependence of the tryptophan emission spectrum, P190 was dissolved at 5 μg/mL in the same buffers described above for CD measurements. For measurements in the presence of 6 M GdnHCl, P190 was allowed to equilibrate for 30 min.

ANS binding to P190

ANS fluorescence emission spectra were measured in the presence and absence of P190, using an excitation wavelength of 372 nm, and emission spectra were scanned from 400 to 600 nm. P190 (0.25 μM) was dissolved in the same pH buffers used for CD spectroscopy in the presence of 10 μM ANS. The resultant spectrum of P190–ANS was obtained by subtracting the signal arising from ANS in the absence of protein from that measured in the presence of P190.

Acknowledgments

This study was supported by grant GM18457 from the National Institutes of Health. We thank J.B. Heymann for helpful discussions and V. Livingston for skilled assistance on this manuscript.

References

Brunden KR, Uratani Y, Cramer WA. 1984. Dependence of the conformation of a colicin E1 channel-forming peptide on acidic pH and solvent polarity. *J Biol Chem 259*:7682–7687.

Bychkova VE, Pain RH, Ptitsyn OB. 1988. The "molten globule" state is involved in the translocation of proteins across membranes? *FEBS Lett 238*:231–234.

Cramer WA, Cohen FS, Merrill AR, Song HY. 1990. Structure and dynamics of the colicin E1 channel. *Mol Microbiol 4*:519–526.

Davidson VL, Brunden KR, Cramer WA. 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.

Dolgikh DA, Gilmanshin RI, Brazhnikov EV, Bychkova VE, Semisotnov GV, Venyaminov S Yu, Ptitsyn OB. 1981. α-Lactalbumin: Compact state with fluctuating tertiary structure? *FEBS Lett 136*:311–315.

Duché D, Parker MW, Gonzalez-Mañas JM. 1994. Uncoupled steps of the colicin A pore formation demonstrated by disulfide bond engineering. *J Biol Chem 269*:6332–6339.

Elkins PA, Song HY, Cramer WA, Stauffacher CV. 1994. Crystallization and characterization of colicin E1 channel-forming polypeptides. *Proteins Struct Funct Genet 19*:150–157.

Haynie DT, Freire E. 1993. Structural energetics of the molten globule state. *Proteins Struct Funct Genet 16*:115–140.

Jagannadham MV, Balasubramanian D. 1985. The molten globular intermediate form in the folding pathway of human carbonic anhydrase B. *FEBS Lett 188*:326–330.

Jiang JX, London E. 1990. Involvement of denaturation-like changes in *Pseudomonas* exotoxin A hydrophobicity and membrane penetration determined by characterization of pH and thermal transitions. Roles of two distinctly conformationally altered states. *J Biol Chem 265*:8636–8641.

Kim PS, Baldwin RL. 1990. Intermediates in the folding reactions of small proteins. *Annu Rev Biochem 59*:631–660.

Lakey JH, van der Goot FG, Pattus F. 1994. All in the family: The toxic action of pore-forming colicins. *Toxicology 87*:85–108.

Lau A, McLaughlin A, McLaughlin S. 1981. The adsorption of divalent cations to phosphatidylglycerol bilayer membranes. *Biochim Biophys Acta 645*:279–292.

Lohner K, Esser AF. 1991. Thermal unfolding and aggregation of human complement protein C9: A differential scanning calorimetry study. *Biochemistry 30*:6620–6625.

London E. 1992. How bacteria protein toxins enter cells: The role of partial unfolding in membrane translocation. *Mol Microbiol 6*:3277–3282.

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

Merrill AR, Cohen FS, Cramer WA. 1990. On the nature of the structural change of the colicin E1 channel peptide necessary for its translocation-competent state. *Biochemistry 29*:5829–5836.

Muga A, Gonzalez-Mañas JM, Lakey JH, Pattus F, Surewicz WK. 1993. pH-dependent stability and membrane interaction of the pore-forming domain of colicin A. *J Biol Chem 268*:1553–1557.

Muga A, Mantsch HH, Surewicz WK. 1991. Membrane binding induces destabilization of cytochrome *c* structure. *Biochemistry 30*:7219–7224.

Ohgushi M, Wada A. 1983. "Molten-globule state": A compact form of globular proteins with mobile side-chains. *FEBS Lett 164*:21–24.

Pain R. 1991. A case of a flexible friend. *Nature 354*:353–354.

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

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

Peterson AA, Cramer WA. 1987. Voltage-dependent monomeric channel ac-

tivity of colicin E1 in artificial membrane vesicles. *J Membr Biol 99*:197–204.

Ptitsyn OB. 1992. The molten globule state. In: Creighton TE, ed. *Protein folding*. New York: W.H. Freeman and Co. pp 243–300.

Ramsay G, Montgomery D, Berger D, Freire E. 1989. Energetics of diphtheria toxin membrane insertion and translocation: Calorimetric characterization of the acid pH induced transition. *Biochemistry 28*:529–533.

Semisotnov GV, Rodionova NA, Razgulyaev OI, Uversky VN, Gripas AF, Gilmanshin RI. 1991. Study of the "molten globule" intermediate state in protein folding by a hydrophobic fluorescent probe. *Biopolymers 31*:119–128.

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

Shin YK, Levinthal C, Levinthal F, Hubbell WL. 1993. Colicin E1 binding to membranes: Time-resolved studies of spin-labeled mutants. *Science 259*:960–963.

Skolnick J, Kolinski A, Godzik A. 1993. From independent modules to molten globules; observations on the nature of protein folding intermediates. *Proc Natl Acad Sci USA 90*:2099–2100.

Stryer L. 1965. The interaction of a naphthalene dye with apomyoglobin and apohemoglobin: A fluorescent probe of non-polar binding sites. *J Mol Biol 13*:482–495.

van der Goot F, Gonzalez-Mañas JM, Lakey JH, Pattus F. 1991. A "molten-globule" membrane-insertion intermediate of the pore-forming domain of colicin A. *Nature 354*:408–410.

van Dijck PWM, de Kruijff B, Verkleij AJ, van Deenen LLM, de Gier J. 1978. Comparative studies on the effects of pH and Ca^{2+} on bilayers of various negatively charged phospholipids and their mixtures with phosphatidylcholine. *Biochim Biophys Acta 512*:84–96.

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

Zakharov SD, Zhang YL, Heymann JB, Cramer WA. 1995. Colicin E1 ion channel binding with membrane: An optimum electrostatic interaction is required for activity. *Biophys J 68*. Forthcoming. [Abstr.]

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

Zhao JM, London E. 1986. Similarity of the conformation of diphtheria toxin at high temperature to that in the membrane-penetrating low pH state. *Proc Natl Acad Sci USA 83*:2002–2006.