Sibbet, G. J., Carpenter, B. G., Ibel, K., & May, R. P. (1983) *Eur. J. Biochem. 133*, 393–398.
Simon, R. H., Camerini-Otero, R. D., & Felsenfeld, G. (1978) *Nucleic Acids Res. 5*, 4805–4818.
Sterner, R., Boffa, L. C., Chen, T. A., & Allfrey, V. G. (1987) *Nucleic Acids Res. 15*, 4375–4391.
Stockley, P. G., & Thomas, J. O. (1979) *FEBS Lett. 99*, 129–135.
Thomas, J. O., & Kornberg, R. D. (1975) *Proc. Natl. Acad. Sci. U.S.A. 72*, 2626–2630.
Thomas, J. O., & Oudet, P. (1979) *Nucleic Acids Res. 7*, 611–623.
Uberbacher, E. C., & Bunick, G. J. (1985) *J. Biomol. Struct. Dyn. 2*, 1033–1055.
Uberbacher, E. C., Ramakrishnan, V., Olins, D. E., & Bunick, G. J. (1983) *Biochemistry 22*, 4916–4923.
van Holde, K. E. (1989) *Chromatin*, Springer-Verlag, New York.
Walker, I. O. (1984) *Biochemistry 23*, 5622–5628.
Wong, N. T. N., & Candido, E. P. M. (1978) *J. Biol. Chem. 253*, 8263–8268.
Wu, R. S., Panusz, H. T., Hatch, C. L., & Bonner, W. M. (1986) *CRC Crit. Rev. Biochem. 20*, 201–263.
Yager, T. D., & van Holde, K. E. (1984) *J. Biol. Chem. 259*, 4212–4222.
Yager, T. D., & McMurray, C. T., & van Holde, K. E. (1989) *Biochemistry 28*, 2271–2281.

On the Nature of the Structural Change of the Colicin E1 Channel Peptide Necessary for Its Translocation-Competent State†

A. R. Merrill,‡ F. S. Cohen,§ and W. A. Cramer*,‡

Department of Biological Sciences, Purdue University, West Lafayette, Indiana 47907, and Department of Physiology, Rush Medical College, Chicago, Illinois 60612

Received May 24, 1989; Revised Manuscript Received February 13, 1990

ABSTRACT: Acidic pH conditions required in vitro for membrane binding and activity of the channel-forming colicin E1 resulted in an increased susceptibility to proteases of the 178-residue thermolytic channel peptide, an increased accessibility to acrylamide of a fluorescence probe linked to cysteine-505 of the peptide, and an increased partition into nonionic detergent. The structural change in the peptide sensed by the fluorescence probe caused by a transition from pH 6.0 to 3.5 occurred in <1 s. The presence of low concentrations of detergents (0.001% SDS or 0.44% octyl β-D-glucoside) or urea (0.2 M) at pH 6 or 4 also increased the susceptibility of the channel peptide to proteases. The increase in protease susceptibility and acrylamide accessibility at low pH, as well as partition of the peptide into nonionic detergent, suggested that acidic pH or the detergents might cause peptide unfolding. However, the hydrodynamic radius of the channel peptide at pH 6, 21–23 Å, was not changed at pH 3.5 or by detergents or urea under conditions that increased the susceptibility of the peptide to protease. The activity of the channel peptide at pH 6 measured with liposomes and planar bilayers, which was a factor of 10^3–10^4 smaller than that at pH 4, was increased by 2–4 orders of magnitude by 0.001% SDS or 0.44% octyl β-D-glucoside, with an additional small increment of activity on planar bilayers caused by 0.01% SDS. A small increase in Stokes radius of the peptide in the presence of SDS could be detected that was approximately correlated with increased activity.

Colicin E1 is a bactericidal protein whose cytotoxic action results from formation of a channel in the cytoplasmic membrane sufficiently conductive to depolarize and deenergize the cell (Gould & Cramer, 1977; Schein et al., 1978; Bullock et al., 1983; Cleveland et al., 1983). The prerequisites for channel formation are binding of the colicin molecule to a receptor in the outer membrane, translocation across this membrane, and binding, import, and channel formation in the cytoplasmic membrane. The molecular events associated with colicin channel formation in the cytoplasmic membrane can be mimicked in vitro by using artificial planar bilayer membranes [e.g., Bullock et al. (1983)] and membrane vesicles (Peterson & Cramer, 1987).

Translocation competence of proteins that are imported or secreted across the membranes of organelles and bacterial cells is believed to require an "unfolded" conformation. The definition of this state is mainly based on the following observations: (i) Mitochondrial import of a fusion protein of cytochrome oxidase subunit IV with dihydrofolate reductase is inhibited if the native folded structure of reductase is stabilized with respect to protease digestion by the compound methotrexate, which binds to its active site (Eilers & Schatz, 1986). (ii) Nascent pre maltose-binding protein that is competent for membrane assembly is also sensitive to protease digestion, whereas the protein loses translocation competence as it folds into a mature protease-resistant structure (Randall & Hardy, 1986). Although these protease data are consistent with an unfolded state, there is a paucity of physical–chemical information concerning the characterization of this state.

The in vitro activity of colicin E1 requires an acidic pH with an optimum ≤4.0 (Davidson et al., 1985). The requirement of an acidic pH for optimum activity is shared by the channel-forming colicin A (Lazdunski et al., 1988), several toxins

† This work was supported by grants from the NIH (GM-18457) and the Markey Foundation to W.A.C., by NIH Grant GM-27367 (F.S.C.), and by an NSERC Fellowship from the government of Canada (A.R.M.).
* To whom correspondence should be addressed.
‡ Purdue University.
§ Rush Medical College.

0006-2960/90/0429-5829$02.50/0

such as diphtheria toxin (Draper & Simon, 1981; Sandvig & Olsnes, 1981; Kagan et al., 1981) and *Pseudomonas* exotoxin A (Farabaksh & Wisnieski, 1989), and proteins of both enveloped and nonenveloped viruses (Wiley & Skehel, 1987). In the case of the toxins, it also has been argued that an important effect of low pH in conveying high activity in vitro and in vivo is an associated unfolding of the protein. This has been inferred from the following observations: (i) the partition of the several toxins, diphtheria, *Pseudomonas*, and *Shigella*, into nonionic detergent at low pH, indicating greater exposure of the hydrophobic interior of the initially water-soluble toxins (Sandvig & Olsnes, 1981; Sandvig & Brown, 1987; Sandvig & Moskaug, 1987), and (ii) for diphtheria toxin, increased solvent exposure of tryptophan residues (Blewitt et al., 1985) and an abrupt decrease below pH 5 in the enthalpy of unfolding and the denaturation transition temperature (Ramsay et al., 1989). The nature of this unfolded state has been described as "denaturation-like" (Chung & London, 1988) and the unfolding transition as "massive" (Ramsay et al., 1989).

The present work documents that acidic pH, or a low concentration of detergent (Bullock & Cohen, 1986) at pH 6, both of which convey high in vitro activity to the colicin channel peptide, establishes a different structural state that could be defined as unfolded by several experimental criteria that have been applied to protein translocation in organelles, bacteria, and toxins. The hydrodynamic properties of this state indicate that no unfolding of the colicin channel peptide can be detected at low pH, although a small degree of unfolding could be correlated with the increase of translocation competence in the presence of low concentrations of SDS.[1]

Materials and Methods

Purification of Colicin E1 and COOH-Terminal Thermolytic Peptide. Colicin E1 was purified from JC411 cells harboring pDMS630, as described previously (Cleveland et al., 1983), except that 0.1 M potassium phosphate buffer, pH 7.0, was used for the final dialysis. Alternatively, for some experiments, colicin E1 was purified from an *Escherichia coli* K-12 strain, DM1187 [from I. Tessman (Tessman et al., 1978)], that has a constitutive SOS repair system. In the presence of a *kil*$^-$ derivative of the ColE1 plasmid, pMY440 (from K. Shirabe), the strain can be stably maintained and colicin E1 production is increased 5–10-fold over previous levels. Briefly, an inoculum of frozen cell stock was placed into 4 × 1 L of 2× BactoTryptone–yeast extract medium and grown for 15 h at 37 °C. These cells were harvested and disrupted, and the 174–178-residue COOH-terminal thermolytic peptide of colicin E1 (approximately half NH_2-Ile-345 and the other half NH_2-Ala-348 and NH_2-Val-349) was prepared as in Shiver et al. (1987).

Preparation of [^{3}H]Thermolytic Fragment. Colicin E1 was tritiated as described previously (Davidson et al., 1985). The [^{3}H]thermolytic peptide was prepared from [^{3}H]colicin by the method of Shiver et al. (1987).

Preparation of IAEDANS–Colicin Peptide Adduct. The COOH-terminal thermolytic peptide of colicin E1 was concentrated to 40 mg/mL in 0.1 M sodium phosphate buffer by centrifugation (Centricon, molecular weight cutoff 10 000; Amicon, Danvers, MA), from which 0.1 mL, pH 7.0, was added to 0.9 mL of 6 M guanidine hydrochloride and 50 mM Tricine buffer, pH 8.0, containing 4 mg of the fluorescence probe IAEDANS, 5-[[[(iodoacetyl)amino]ethyl]amino]-naphthalene-1-sulfonic acid (Molecular Probes Inc., Eugene, OR). This mixture was mixed and subsequently reacted in the dark at 4 °C for 24 h. The yellow solution was diluted to 4.0 mL with 50 mM Tricine buffer, pH 8.0, containing 5.4 M guanidine hydrochloride, and then dialyzed in the dark against 0.1 M sodium phosphate buffer, pH 7.0 (1000-fold dilution), for 8–10 h. The resulting dialyzate was filtered through a disposable Millipore filter (pore size, 0.2 μm), and the absorbance spectrum of the filtrate was used to determine the stoichiometry of the reaction. ϵ_M (M^{-1} cm^{-1}) values of 3.06 × 10^4 (280 nm) and 6 × 10^3 (340 nm), respectively, were used for determination of peptide concentration (Brunden et al., 1984) and the concentration of conjugated IAEDANS (Hudson & Weber, 1973). Typically, the yield of labeled protein was 30% and the stoichiometry of IAEDANS:peptide was 0.8–0.9 (mol:mol), indicating reaction with the only cysteine (Cys-505) in the peptide. The IAEDANS did not react with the peptide in the absence of a denaturant, consistent with previous studies on the inaccessibility of Cys-505 (Bishop et al., 1986). Neither the in vitro activity of the colicin peptide adduct nor its cytotoxicity measured with osmotically shocked cells decreased relative to the unmodified peptide, so that IAEDANS could be used as a probe of pH-dependent structure changes.

Proteolysis of Colicin COOH-Terminal Peptide. Colicin channel peptide, 1.0 mg/mL, was subjected to proteolysis at a ratio of 1:1 (w:w peptide:protease) with papain and bromelain at 25 °C for 60 min (data of Figure 1) or with Pronase E (Sigma, Type XXV, lot no. 56F-0635) at 25 °C for 10 min (data of Figure 2). The peptide was treated with 0.001% (34 μM) SDS, 15 mM octyl β-glucoside, or 0.2 M urea (final concentration) by incubating the protein in the appropriate denaturing solution for 30 min, followed by the addition of Pronase E in 100 mM $NaNO_3$, 10 mM DMG, and 0.5 mM $CaCl_2$, pH 4.0 or 6.0 (1:1 w:w peptide:Pronase) at 25 °C. For the experiments with papain and bromelain, the proteolysis was terminated by precipitating the protein with TCA to inactivate the protease, followed by the addition of buffer (Laemmli, 1970) and subsequent electrophoresis on a Pharmacia Phast Gel system (Pharmacia-LKB, Sweden). For experiments involving Pronase E, only bromophenol blue was added to the samples (no SDS) prior to electrophoresis. This was done to avoid any additional proteolysis that could have occurred upon addition of Laemmli buffer to the samples. The omission of SDS from the sample buffer had no effect on the electrophoretic migration of the proteins.

Size-Exclusion Chromatography and Determination of Stokes Radius of Channel Peptide. Size-exclusion chromatography was performed as described by Andrews (1970) with a Superose 12 column (1.0-cm diameter × 25-cm length) attached to a Pharmacia FPLC system, using a solution of 20 mM DMG and 150 mM NaCl, pH 6.0, at a flow rate of 0.5 mL/min to develop the column. Standard proteins, contained in a Pharmacia molecular weight kit, were used to calibrate the column. These proteins included ribonuclease A (R_S, 16.4 Å), chymotrypsinogen A (R_S, 20.9 Å), ovalbumin (R_S, 30.5 Å), albumin (R_S, 35.5 Å), aldolase (R_S, 48.1 Å), catalase (R_S, 52.2 Å), ferritin (R_S, 61.0 Å), and thyroglobulin (R_S, 85.0 Å). To test the effects of SDS on R_S, under conditions that mimicked those used to alter protease susceptibility, and on activity,

[1] Abbreviations: ANS, 1-anilinonaphthalene-8-sulfonic acid; cmc, critical micelle concentration; CoIV–DHRF, fusion protein of cytochrome oxidase subunit IV leader peptide and dihydrofolate reductase; DMG, dimethyl glutarate; DOPC, dioleoylphosphatidylcholine; DOPG, dioleoylphosphatidylglycerol; ϵ_M, molar extinction coefficient; IAEDANS, 5-[[[(iodoacetyl)amino]ethyl]amino]naphthalene-1-sulfonic acid; K_{SV}, Stern–Volmer quenching constant; R_S, Stokes radius; SDS, sodium dodecyl sulfate; TCA, trichloroacetic acid.

1 mg/mL peptide and 10 μg/mL peptide, respectively, were incubated with different concentrations of SDS for 30 min at 25 °C. The column was equilibrated with the particular concentration of SDS. The peptide was found to be diluted approximately 10-fold upon elution from the column.

In Vitro Channel Activity; Cl^- Efflux from Artificial Vesicles. Channel-forming activities of native thermolytic peptide and the IAEDANS–peptide conjugate were determined by protein-mediated Cl^- efflux from large, unilamellar vesicles [lipid:peptide (mol:mol) = 2×10^6:1], prepared from asolectin as described previously (Peterson & Cramer, 1987). Cl^- efflux was measured with an ion-specific electrode (Orion 94-17B) and a double-junction reference calomel electrode (Orion 90-02).

Assay of Vesicle Intactness with Carboxyinulin. Peptide, with or without detergent treatment (0.1% SDS or 15 mM octyl glucoside, 30 min at 25 °C), was added to [^{14}C]-carboxyinulin-loaded asolectin liposomes in the presence of a membrane potential; the final protein concentration was 6 ng/mL [lipid:protein (mol:mol) = 3×10^5:1]. The final detergent concentrations were 4.3×10^{-5}% and 1.1×10^{-4}% for SDS and octyl β-glucoside, respectively. The mixture was allowed to incubate for 5 min at 25 °C and then centrifuged at 30000*g* for 20 min at 4 °C to sediment the liposomes. An aliquot of the supernatant was removed and the amount of [^{14}C]carboxyinulin released was determine by liquid scintillation counting.

Peptide Partition into Nonionic Detergent. Binding of [^{3}H]peptide to the nonionic detergent Triton X-114 as a function of pH was assayed essentially by the method of Bordier (1981). The Triton was repurified by three overnight phase separations at 30 °C to remove water-soluble impurities. Labeled peptide (20 μg) was mixed at the appropriate pH with 50 mM DMG buffer, 150 mM NaCl, and 1% Triton X-114 and incubated for 1 h on ice at 0 °C, after which the mixture (0.2-mL total volume) was removed from the Eppendorf tube and layered on a sucrose cushion (0.3 mL of a 6% sucrose, 0.006% Triton X-114, 150 mM NaCl, and 10 mM DMG solution). This sample was placed in a water bath at 30 °C for 3 min followed by centrifugation in a swinging bucket microcentrifuge at 300*g* for 3 min. The sediment in the bottom of the Eppendorf tube contains all of the original Triton (Bordier, 1981). The upper layer above the sucrose cushion was removed, Triton X-114 was again added to 1% and the solution was incubated for 1 h on ice and then added to the original tube containing the sucrose cushion. After centrifugation, the incubation mixture was removed along with the sucrose cushion, leaving the two combined sediments of concentrated detergent (~30 μL) in the bottom of the microcentrifuge tube. The Eppendorf tube containing the combined sediments was then cut in half, and the lower half was placed in a 20-mL scintillation vial to which 10 mL of aqueous counting scintillant (Amersham) was added for determination of radioactivity.

Planar Lipid Bilayer Measurements. High-capacitance, solvent-free planar bilayers were formed after thinning was completed (Niles et al., 1988) by sequentially raising phospholipid monolayers over a hole coated with squalene (Montal & Mueller, 1972). Other details are as in Shiver et al. (1987). The voltages indicated were those of the trans compartment, and the cis compartment was set equal to 0 mV.

Fluorometry and Spectrophotometry. Fluorescence measurement of IAEDANS and IAEDANS–peptide conjugate were carried out by using a Perkin-Elmer MPF-4 spectrofluorometer equipped with a magnetic stirrer and a thermostated (20 °C) cuvette holder. The excitation and emission wavelengths were at 337 and 475 nm, respectively, with half-bandwidths of 5 nm, and the latter was calibrated by using the 436.8- and 546.1-nm emission lines of a low-pressure Hg lamp. For the quenching experiments, small aliquots of a 6.7 M filtered solution of acrylamide (BRL, ultrapure grade) were added to buffered solution at various pH values containing 2 μM IAEDANS–peptide in 20 mM DMG, 100 mM NaCl, and 0.5 mM $CaCl_2$ and mixed for 1 min. Absorbance spectra of native thermolytic peptide and the IAEDANS–peptide adduct were recorded with a Uvikon Model 810 spectrophotometer.

Determination of Critical Micelle Concentration of SDS. The cmc of the anionic surfactant SDS was determined through the dependence of the fluorescence intensity of the probe ANS (10 μM) on SDS concentration (Vendittis et al., 1981) in 50 mM sodium nitrate and 1 mM potassium phosphate buffer, pH 7.0 (25 °C; excitation, 370 nm, 5-nm band-pass; emission, 480 nm, 10-nm band-pass).

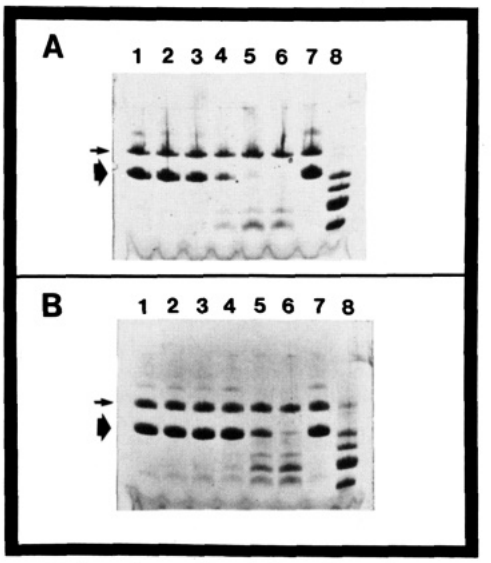


FIGURE 1: pH dependence of sensitivity of colicin channel peptide to protease. Lanes 1–6, peptide incubated with papain (A) and bromelain (B) at 37 °C for 60 min at all pH values except pH 3 in lane 7 (zero time). Lanes: 1, pH 6.0; 2, pH 5.0; 3, pH 4.5; 4, pH 4.0; 5, pH 3.5; 6, pH 3.0; 7, pH 3.0, peptide precipitated immediately after addition of protease; 8, molecular weight standards [(top to bottom) myoglobin polypeptide backbone (17000), myoglobin fragment I–II (14000), myoglobin fragment I (8000), myoglobin fragment II (6000), and myoglobin fragment III (2500)]. The bold and thin arrows indicate the positions of colicin channel peptide and protease, respectively.

RESULTS

(1) pH Dependence of Protease Accessibility/Susceptibility of the Colicin E1 Channel Peptide. The sensitivity to protease of proteins in a putative precursor state for export has been used to infer the existence of an unfolded conformation in this state (Eilers & Schatz, 1986; Randall & Hardy, 1986). The protease sensitivity of the colicin E1 channel peptide at an acidic pH of 3.5 compared to pH 6.0 was probed with papain and bromelain (Figure 1, panels A and B, respectively), two proteases with measurable activity at pH 3.5. The rate of autoproteolysis, as seen in these gels, or of the proteolysis of hemoglobin as a model substrate (Drapeau, 1976) shows that the activity of these proteases either decreases with lowered pH or remains approximately constant. The proteolytic rates of urea-denatured hemoglobin determined for these enzymes at pH 3.5 and pH 6.0, respectively, were (units/mg) 33 and 32 for papain and 25 and 23 for bromelain. Lanes 1–6 in Figure 1 show the increasing effect of protease activity on the

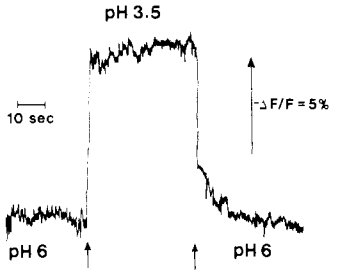


FIGURE 2: Time course of reversible change in fluorescence emission spectrum caused by pH shift. The pH was shifted from 6.0 to 3.5 by the addition of 8 μL of 6 N HCl to 2 mL of pH 6 buffer and from 3.5 to 6.0 by the addition of 8 μL of 6 N NaOH. Buffer composition: 20 mM dimethyl glutarate, 100 mM NaCl, 0.5 mM $CaCl_2$. Excitation wavelength, 337 nm; half-bandwidth, excitation and emission, 5 nm; IAEDANS–peptide concentration, 2 μM.

channel peptide (large dark arrow) at pH values of 6.0, 5.0, 4.5, 4.0, 3.5, and 3.0. The second band seen on the gels at a higher M_r value is the protease (smaller arrow). For papain, there is extensive cleavage of the channel peptide at pH 4.0 and below (Figure 1A, lanes 4–6), but for bromelain, no digestion can be seen until the pH is decreased to 3.5 (Figure 1B, lanes 5 and 6). Lane 7 in both panels shows a control at pH 3.0 (t = 0, no incubation time before TCA precipitation and electrophoresis, no digestion), showing that the protease was inactivated by the TCA before electrophoresis. The increased effect of both proteases with decreasing pH suggests that the peptide may be in a more unfolded state (Eilers & Schatz, 1986; Randall & Hardy, 1986) at pH 4.0 or lower.

(2) IAEDANS–Cys-505 as a Fluorescence Probe of Structure Changes Caused by Acidic pH; pH Dependence of Probe Accessibility to Acrylamide. The increase in peptide susceptibility to the proteases at low pH was similar to that seen by using a fluorescence probe. The uncharged 35-residue segment Ala-474–Ile-508 possesses the only cysteine (Cys-505) in the molecule (Yamada et al., 1982). Cys-505 was labeled with a sulfhydryl-reactive fluorescence probe, IAEDANS, whose fluorescence emission maximum is known to be dependent upon solvent polarity, exhibiting a blue shift in the emission spectrum and an increase in intensity when the polarity is decreased (LaPorte et al., 1981).

The fluorescence emission spectrum of free IAEDANS in aqueous solvent was not affected in the pH range 3.5–8.0. The emission peak of an IAEDANS–cysteine adduct in ethanol, methanol, and water was 457, 468, and 499 nm, respectively, and in liposomes (DOPC/DOPG) at pH 3.5 the emission peak was 460 nm. The emission peak of the peptide adduct in aqueous solution was 475 nm at pH 6.0, indicating that the average local environment around the probe is somewhat more polar than that of methanol. This is consistent with the position of this residue (exposed to solvent near the COOH end of helix 9) predicted from the crystal structure of the colicin A channel peptide (Parker et al., 1989). The IAEDANS–peptide emission peak was red-shifted by 5 nm, to 480 nm, when the pH was decreased from 6.0 to 3.5, indicating a small further increase in the average polarity of the environment of Cys-505 (data not shown). The conformational change sensed by the IAEDANS when the pH is rapidly shifted from 6.0 to 3.5 was measured at an emission wavelength of 475 nm and found to be rapid ($t_{1/2} \leq 0.7$ s, the mixing time within the cuvette) and reversible (Figure 2).

Acrylamide quenching of IAEDANS fluorescence was used as a probe of the solvent accessibility of Cys-505. The quenching of the IAEDANS fluorescence in the adduct was measured as a function of acrylamide concentration at pH values ranging from 6.0 to 3.0 (Table I). The summary of Stern–Volmer Quenching constants (K_{SV}) for acrylamide quenching of free IAEDANS and IAEDANS–peptide adduct as a function of pH (Table I) shows that an abrupt change in the quenching constant occurred when the pH was lowered from 4.0 (K_{SV} = 1.00) to 3.5 (K_{SV} = 1.55), but no further increase in the quenching constant occurred at pH 3.0. The K_{SV} value for the free IAEDANS–cysteine adduct does not change markedly between pH 3.5 and 6.0 (5.20 and 5.15, respectively), indicating that the effect of low pH was on the conformation of the colicin E1 peptide and was not due to altered fluorescence properties of the probe. The quenching of the IAEDANS fluorescence by acrylamide was suppressed when the peptide was incorporated into liposomes at pH 3.5 (data not shown), compared to the unbound peptide at pH 6 or 3.5, showing that when the colicin channel peptide binds to and partially inserts into the membrane, Cys-505 is not accessible from the aqueous phase. To test the role of low pH in generating an unfolded conformation, the effect of other agents expected to perturb the native structure on activity and protease susceptibility was examined.

Table I: Accessibility of IAEDANS–Cysteine/IAEDANS–Peptide to the Neutral Quencher Acrylamide at Various pH Values[a]

fluorescent group	pH	K_{SV} (M^{-1})	$1/K_{SV}$ (M)
IAEDANS–cysteine	3.5	5.20	0.20
IAEDANS–cysteine	6.0	5.15	0.20
IAEDANS–peptide	3.0	1.45	0.70
IAEDANS–peptide	3.5	1.55	0.65
IAEDANS–peptide	4.0	1.00	1.00
IAEDANS–peptide	5.0	0.99	1.01
IAEDANS–peptide	6.0	0.97	1.03

[a] K_{SV} values were determined from the slopes of plots of F_0/F, the ratio of the fluorescence intensity in the absence to that in the presence of quencher, Q, versus [Q] according to the Stern–Volmer equation: $F_0/F = 1 + K_{SV}[Q]$. Excitation wavelength (half-bandwidth) 337 nm (5 nm); emission wavelength (half-bandwidth) 475 nm (5 nm); temperature 20 °C.

(3) Effect of Structural Perturbants on Accessibility to Protease. Sodium dodecyl sulfate, used above its cmc, is commonly used to denature proteins and as such would be expected to be a stronger denaturant than octyl β-glucoside, which is used frequently to stabilize biological activity of membrane proteins. It was found that both detergents caused an increase in sensitivity of the peptide to protease. Incubation of the peptide (1 mg) in dilute SDS (0.001–0.04%, 33 μM–1.3 mM; cmc, 0.06%; SDS:peptide ≈1 at 0.001% SDS) or octyl β-glucoside (0.44%, 15 mM; cmc, 20–25 mM) increased the peptide sensitivity to protease (Pronase E), as shown in Figure 3. This increase in proteolysis was observed in the presence of 0.2 M urea (lanes 5 and 6), 0.44% octyl glucoside (lanes 7 and 8), or 0.001% SDS (lanes 9 and 10) under conditions where the untreated channel peptide was not affected by the protease (Figure 3, lanes 2–4). In particular, the control shown in lane 3 for a sample incubated at pH 4 with Pronase in the absence of SDS shows that the effect of SDS occurred during the preincubation period and not during the running time on the gel. Thus, it might be inferred that these detergents also caused an increase in the degree of unfolding of the channel peptide.

(4) Partition of Peptide into Detergent at Low pH as a Probe of Structure Change. Partition of several toxin molecules into the nonionic detergent Triton X-114 has been used to infer a conformational change at low pH that exposes a hydrophobic domain (Sandvig & Olsnes, 1987; Sandvig & Moskaug, 1987). Partition of the colicin E1 channel peptide into Triton X-114 increased monotonically as the pH was

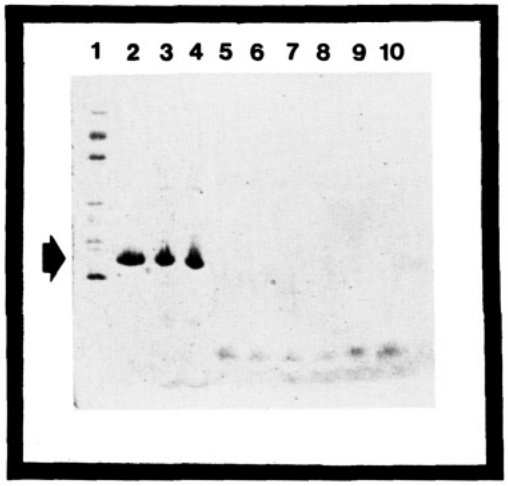


FIGURE 3: Proteolysis of colicin thermolytic peptide in the presence of a detergent or urea. Peptide (1 μg/lane) was incubated with Pronase E (1 mg of peptide, peptide:protease 1:1 w:w) and digestion was allowed to proceed for 15 min at 25 °C. Lanes: 1, molecular weight standards [(top to bottom) phosphorylase B (92 500), BSA (66 000 kDa), ovalbumin (45 000), carbonic anhydrase (31 000), soybean trypsin inhibitor (21 500), and lysozyme (14 400)]; 2, peptide without Pronase; 3, peptide/Pronase, pH 4; 4, peptide/Pronase, pH 6; 5, peptide in 0.2 M urea/Pronase, pH 4; 6, peptide in 0.2 M urea/Pronase, pH 6; 7, peptide in 0.44% octyl β-glucoside/Pronase, pH 4; 8, peptide in 0.44% octyl β-glucoside/Pronase, pH 6; 9, peptide incubated with 0.001% SDS/Pronase, pH 4; 10, peptide incubated with 0.001% SDS/Pronase, pH 6.

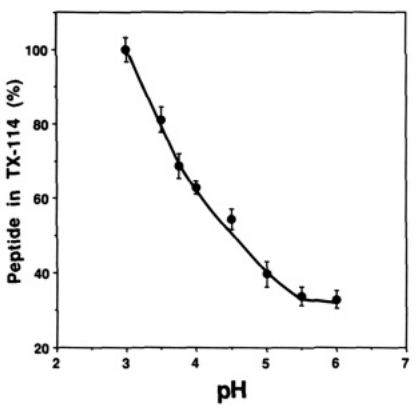
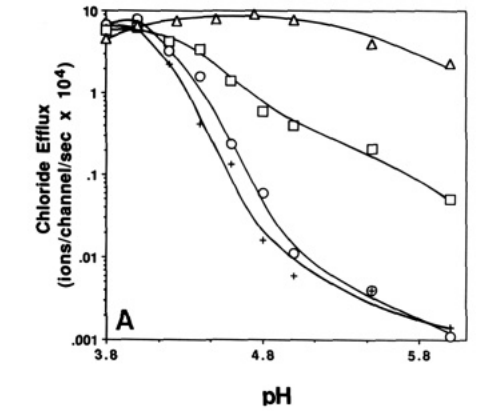


FIGURE 4: Effect of pH on the partition of [^{3}H]peptide into the nonionic detergent Triton X-114. The 100% value at pH 3.0 is equivalent to 1.2×10^4 cpm of [^{3}H]peptide bound to the detergent and has been normalized to 100% from the measured value of 84%.

decreased from 6 to 3 until the partition into the detergent was virtually complete at the latter pH (Figure 4), showing that the peptide assumed a net hydrophobic character at acidic pH. The relation of the structure change measured by this partition experiment to that assayed by protease and fluorescence probe accessibility is not obvious, because the partition function has a less cooperative pH dependence (see Discussion) and significant partition into detergent occurs at pH 4.5. A much more cooperative partition function than that measured with the channel peptide was obtained with the intact colicin E1 molecule (data not shown). There was little effect of pH on the partition into Triton X-114 of an unrelated soluble protein, bovine serum albumin, which increased from 5% at pH 6 to 13% at pH 3.5 (not shown).

(5) pH Dependence of in Vitro Channel Peptide Activity in Dilute SDS or Octyl β-Glucoside. The in vitro activity of channel peptide (3 μg/mL) incubated in SDS (0.001%, SDS:peptide = 350:1 during incubation; △, Figure 5A) or octyl glucoside (0.44%; □, Figure 5A) before addition to Cl^--loaded membrane vesicles was 2–3 orders of magnitude greater at pH



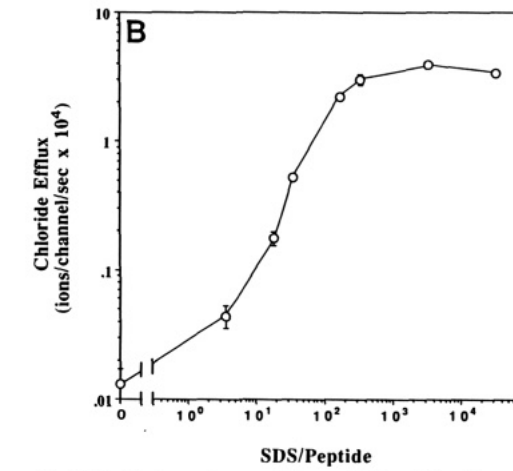


FIGURE 5: (A) pH dependence of the activity of the channel peptide in liposomes after incubation of peptide with a detergent or urea. Liposomes were suspended in 100 mM choline nitrate, 10 mM DMG, and 0.5 mM $Ca(NO_3)_2$ at different pH values, to which peptide with or without detergent or urea was added by a 2000-fold dilution, after an initial 30-min incubation (pH 6, room temperature) in the same buffer under the following conditions: (○) untreated peptide, no detergent; (△) 10^{-3}% (34 μM) SDS, SDS:peptide 350:1 (mol:mol) (ca. 10-fold lower activity was obtained after incubation with 10^{-4}% SDS); (□) 15 mM octyl β-glucoside; (+) 0.2 M urea. Channel activities calculated from initial specific rate of Cl^- efflux. (B) Dependence of activity at pH 6.0 of channel peptide as a function of SDS:peptide ratio. Peptide was incubated (30 min, 25 °C) with SDS, the higher concentrations of peptide being necessary for activity at lower SDS:peptide ratios. Final concentration of peptide added to liposomes (SDS:peptide ratio): 0.1 μg/mL (0–19); 4.5 ng/mL (35); 0.75–4.5 ng/mL (175–35 000).

6.0 compared to that of the untreated peptide (○, Figure 5A). The absolute concentration of the colicin E1 peptide used to measure activity was ≲50 pM (~1 ng/mL). Soluble proteins such as carbonic anhydrase or BSA cause no detectable solute efflux when added to liposomes at these concentrations. The activity in 0.001% SDS was greater than that in octyl glucoside and was essentially pH-independent. The increased activity with SDS–peptide was found not to arise from induced vesicle leakiness or breakage, as tested with entrapped carboxyinulin (average Stokes diameter, 30 Å) (not shown), nor did addition of SDS at the final concentration (5×10^{-7}%) have any effect. Significant efflux of carboxyinulin in the absence of SDS occurred only at peptide:lipid ratios ≳5×10^{-4}, a molar ratio of peptide:lipid 1000 times larger than that typically used, 5×10^{-7}, in Cl^- efflux experiments. The activity of the peptide as a function of the molar ratio of added SDS:peptide is shown in Figure 5B. The activity was increased by the presence of SDS at an SDS:peptide ratio of 3.5, increasing monotonically up to a ratio of 200–300 where the SDS effect saturates.

The correlation between increased accessibility to protease and increased activity at high pH values, both effects caused

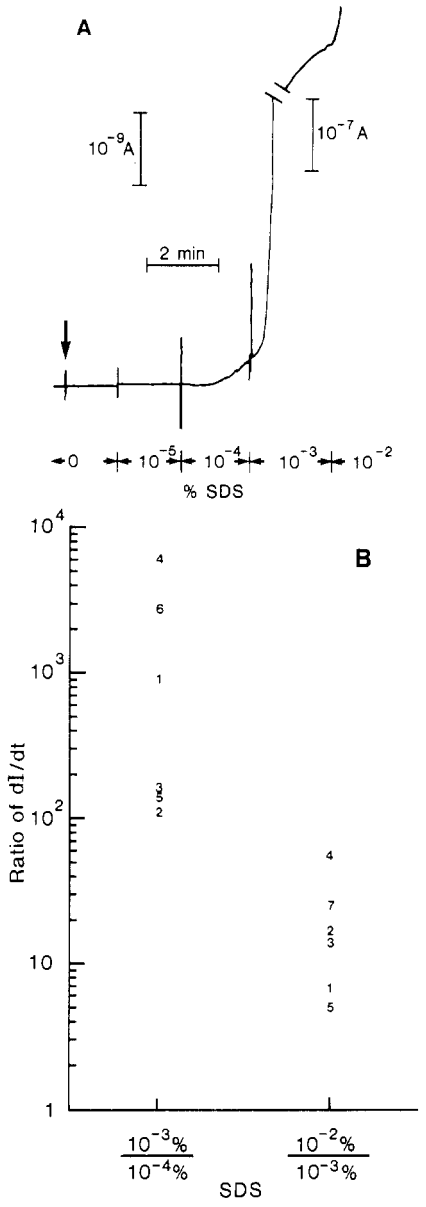


FIGURE 6: (A) Effect of SDS on macroscopic conductance of the colicin E1 channel peptide in planar bilayers. Thermolytic peptide of colicin E1 (600 ng/mL) was added (arrow) to the cis side of an asolectin planar membrane separating symmetrical solutions of 100 mM NaCl, 3 mM $CaCl_2$, 10 mM MES, and 0.1 mM EDTA, pH 6.0. The membrane was voltage-clamped at −20 mV. SDS was added to the cis compartment, bringing its concentration to the indicated values. For this membrane, activity was not observed until the SDS was brought to 10^{-4}%. After the SDS concentration was increased to 10^{-3}%, the gain was reduced 2 orders of magnitude, as indicated by the break mark. (B) The ratio of the dI/dt values measured at a concentration of 10^{-3}% SDS relative to those measured at 10^{-4}% SDS, and of those measured at a concentration of 10^{-2}% to those measured at 10^{-3}% SDS, is shown on a logarithmic scale, derived from experiments illustrated in (A). The values of these ratios are denoted by numbers, with the numbers referring to individual membranes. Thus, points with the same numbers were derived from the same membrane.

by SDS and octyl glucoside, might imply that the increase in activity arises from a general unfolding of the peptide. However, the lack of effect on the activity of dilute (0.2 M) urea (+, Figure 5A), whose presence also resulted in increased protease sensitivity (lanes 9 and 10, Figure 3), was in marked contrast to the effects of SDS and octyl glucoside.

The effect of SDS on channel activity in planar bilayers resembled that in liposomes. No effect was observed on the rate of current change (dI/dt) at pH 6 when 10^{-5}% SDS was added to the cis side of planar membranes to which untreated peptide had been added previously (Figure 6A). However, when the SDS concentration was increased to between 10^{-4}% and 10^{-3}%, dI/dt increased greatly. Further increases in the SDS concentration to 10^{-2}% caused additional increases in dI/dt (Figure 6A). Quantitatively, dI/dt in the presence of 10^{-3}% SDS was 2–4 orders of magnitude greater than in 10^{-4}% SDS, whereas dI/dt for 10^{-2}% SDS was 1–2 orders of magnitude greater than dI/dt observed with 10^{-3}% SDS (Figure 6B). At pH 4, dI/dt did not vary as the SDS concentration was progressively increased in the cis compartment from 0 to 10^{-2}% SDS. This independence of activity at pH 4 is in accord with the same channel activity observed on vesicles in the presence and absence of SDS at this pH (Figure 5).

Table II: Effects of pH, Detergents, and Denaturing Agents on the Stokes Radius of Colicin E1 Thermolytic Peptide

(A) Effect of Denaturing Agents			
agent	concn (M)	R_S[a] (Å)	hw/ph[b]
guanidine hydrochloride	6.0	84.8 ± 0.2	0.32
urea	0.2	22.8 ± 0.4	0.08
urea	6.0	25.4 ± 0.4	0.09
urea	8.0	76.4 ± 0.3	0.31
(B) Effect of Detergents (pH 6.0)			
detergent	concn (%)	R_S (Å)	hw/ph
octyl glucoside	0.44 (15 mM)	22.8 ± 0.4	0.11
SDS	0.01	33.9 ± 5.7[c]	0.52
SDS	0.005	23.0 ± 0.7	0.21
SDS	0.001	22.8 ± 0.4[d]	0.11
SDS	0.0001	22.9 ± 1.1	0.10
(C) Effect of pH (No Detergent)			
pH	R_S (Å)	hw/ph	
3.5	20.7 ± 0.4	0.07	
6.0	22.7 ± 0.3	0.06	

[a] The Stokes radius (R_S) was determined from a plot of the known R_S of standard proteins versus $(-\log K_{av})^{1/2}$ for each protein where $K_{av} = (V_e - V_0)/(V_t - V_0)$. V_e is the elution volume of the protein peak; V_0, void volume of column; V_t, total column volume. Mean values and standard deviations were calculated from four measurements except as noted. [b] hw/ph represents the half-width of the peak of the elution profile divided by the peak height, calculated by using mean values. [c] Mean value and standard deviation were calculated from eight measurements. [d] Mean value and standard deviation were calculated from six measurements.

(6) Hydrodynamic Radius of Colicin Peptide at Low pH and in the Presence of Structural Perturbants. Unfolding of the channel peptide by defined conditions for denaturation caused a large increase in the hydrodynamic (Stokes) radius of the colicin channel peptide, measured by elution from a calibrated FPLC Superose column. The calculated radius of the channel peptide at pH 6.0 was 23 Å and increased to 76 and 85 Å at this pH in the presence of 8 M urea or 6 M guanidine hydrochloride, respectively (Table IIA). In contrast, conditions that were shown to increase protease accessibility (Figure 3) at pH 6 (0.001% SDS, incubated with 1 mg of peptide, SDS:peptide = 1; 0.44% octyl β-D-glucoside) had at most a small effect on the hydrodynamic properties. The average hydrodynamic radius of the peptide remained unchanged at 22–23 Å, corresponding to a molecular weight 24000–26000 for a spherical protein, in the presence of 0.44% octyl glucoside and concentrations of SDS ≤0.001%. A larger hydrodynamic radius was observed with 0.01% SDS. With 0.44% octyl glucoside and 0.001% SDS, the width of the eluted peptide peak (described by its ratio of half-width to peak height) increased slightly (Table IIB) relative to that of the untreated peptide at pH 6.0 (Table IIC). It should be noted that the calculated SDS:peptide ratios (Tables IIB and III) are probably somewhat underestimated by the calculation

Table III: The Stokes Radius of the Colicin E1 Thermolytic Peptide as a Function of Initial SDS:Peptide Ratio

SDS:peptide[a]	R_S[b] (Å)	hw/ph
0	21.5 ± 0.4	0.11
19	21.5 ± 0.5	0.11
35	21.6 ± 0.3	0.12
88	26.1 ± 0.4	0.40
175	27.7 ± 0.8	0.42
350	30.4 ± 0.4	0.50
3500	33.7 ± 0.4	0.72

[a] mol:mol. [b] Mean values and standard deviations were calculated from two measurements.

based on the concentration present during the initial incubation conditions. The peptide is diluted by approximately a factor of 10 on the column while the initial SDS concentration is maintained throughout the column.

The acidic conditions (pH 3.5) that result in greatly increased activity and increased accessibility to protease and acrylamide gave no indication of any unfolding. The measured hydrodynamic radius at pH 3.5, 20.7 Å, decreased slightly relative to that at pH 6.0 (Table IIC).

The Stokes radius was also measured as a function of SDS:peptide ratio (Table III) under conditions mimicking as closely as possible those used for measurement of activity (Figure 5). The Stokes radius of the control peptide at pH 6 was 21.5 Å. No effect of added SDS was seen at an SDS:peptide molar ratio of 35. The average Stokes radius increased to 26 Å at an SDS:peptide ratio of 88 and to 30 Å at a ratio of 350, where the effect on the activity (Figure 5B) was maximal, with accompanying increases in the width of the distribution. These increases in Stokes radius of 5–10 Å are much smaller than those of 55–65 Å that occurred upon complete denaturation with urea or guanidine hydrochloride (Table IIA).

Discussion

Low pH, required for optimum in vitro activity of the colicin E1 channel, has several effects on the peptide: (i) decreased single-channel conductance of colicins A (Pattus et al., 1983) and E1 (Bishop et al., 1986), (ii) increased anion selectivity of the channel (Raymond et al., 1986), (iii) neutralization of acidic amino acid residues that would electrostatically facilitate binding to and entry of the channel into the bilayer (Davidson et al., 1985), and (iv) increased protease susceptibility, acrylamide accessibility, and partition into nonionic detergent, as documented in the present work. Of these effects, (iii) and (iv) involve changes in molecular properties that could contribute to the increase in macroscopic conductance observed at low pH with membrane vesicles and planar bilayers.

The increase in protease accessibility at pH 6 caused by low concentrations of these detergents (Figure 3), the increase in susceptibility to protease at low pH (Figure 1), and the increase in accessibility to acrylamide (Table I) are criteria that are similar to those used to infer that unfolding of a precursor protein is a prerequisite for import in mitochondria (Eilers & Schatz, 1986, 1988) and for secretion in *E. coli* (Randall & Hardy, 1986). The partition into Triton X-114 at low pH (Figure 4) is a criterion that has been used to infer that a pH-dependent exposure of part of the hydrophobic interior is a prerequisite for the action of several toxins on membranes. One difference between partitioning of the colicin E1 channel peptide and intact toxins (Sandvig & Brown, 1987; Sandvig & Moskaug, 1987), compared to intact colicin E1 (Escuyer et al., 1986), into Triton X-114 is that the partition function of the E1 channel peptide, although not of the intact colicin E1, was less cooperative. A larger degree of cooperativity was also seen for the binding of intact colicin E3 to Triton X-100 (Escuyer et al., 1986). The pH dependence of the channel peptide detergent partition function (Figure 4), which increases monotonically between pH 6 and 3, also contrasts with the steeper and more cooperative pH dependence of protease and acrylamide accessibility, where no effect is seen until pH $\lesssim$4–4.5 (Figure 1, Table I). The difference between the pH dependence of the detergent partition function and that of protease and acrylamide accessibility indicates either that they may be reporting different aspects of the pH-dependent structure change or that the different temperatures and times involved in the two sets of assays result in a different pH dependence.

While proteolysis and fluorescence quenching data reported here are similar to those used previously to infer a partial or complete unfolding for the translocation-competent state in import, secretion, and toxin action, some of the data argue against this inference: (i) Urea (0.2 M) caused qualitatively the same increase in protease sensitivity as did 0.001% SDS or 0.44% octyl β-D-glucoside but, unlike the detergents, did not increase peptide activity. This might be ascribed to a lower degree or different kind of unfolding caused by the dilute urea that was not resolved by the protease assay. (ii) Treatment with detergents or urea under conditions that caused an increase in protease or acrylamide accessibility to the peptide did not cause an increase in its hydrodynamic radius. There was no change upon treatment with octyl β-glucoside, 0.001% SDS, or 0.2 M urea in the average hydrodynamic radius, 22–23 Å, of the native peptide, which increased to ~80 Å for the fully denatured peptide. The low pH condition resulted in a slight decrease in hydrodynamic radius and no change in elution bandwidth. Thus, unfolding of the channel peptide caused by acidic pH is not detectable. It was already known that unfolding of the channel peptide at low pH could involve at most the tertiary and not the secondary structure, because the large α-helical content of the peptide ($\gtrsim$50%) measured by circular dichroism showed no tendency to decrease as the pH was lowered to 3.5 in the presence or absence of nonionic detergent (Brunden et al., 1984). It was concluded that the increase in protease susceptibility that is commonly used as a diagnostic indicator of protein unfolding related to translocation competence did not reflect, in the present case, any obvious unfolding of the colicin E1 channel peptide.

The SDS:peptide stoichiometry (~1.0) that caused an increase in protease and acrylamide accessibility (Figure 3, Table I) was very low. The low stoichiometry of SDS:peptide might have been expected to allow limited proteolysis (~63% of the peptide) if the SDS adsorbed randomly and irreversibly. However, as the proteolysis progressed, it is likely that SDS was released from the digested peptide and the ratio of SDS to undigested peptide increased. The higher ratios that affected activity (Figure 4) did cause a small increase in Stokes radius of the peptide (Table III).

The above data lead to the conclusion that the translocation-competent state of the colicin E1 channel at low pH cannot be ascribed to an extensively unfolded state by hydrodynamic criteria. The state of the colicin E1 channel peptide that is competent for activity at low pH cannot be described, as has been done for diphtheria toxin, as denaturation-like (Chung & London, 1988) nor the low pH unfolding transition as massive (Ramsey et al., 1989). It has been noted that circular dichroism and tryptophan fluorescence studies that the low pH conformation of diphtheria toxin is not extensively unfolded (Blewitt et al., 1985). It is also not clear

that the massive unfolding that has been attributed from calorimetry analysis to diphtheria toxin below pH 5–5.5 (Ramsay et al., 1989) has occurred at pH 5.8, where diphtheria toxin can have high activity (Chung & London, 1988). Similarly, the greater sensitivity to protease in the absence of inhibitors of dihydrofolate reductase, the part of the CoIV-DHFR fusion protein that is imported by mitochondria (Eilers & Schatz, 1986), has been attributed to an altered conformation of the protein (Partishall et al., 1976) that is not necessarily unfolded.

The translocation-competent state at low pH is associated with increased susceptibility to protease, increased accessibility of the COOH end of the hydrophobic domain to acrylamide, and increased partition into nonionic detergent. These changes are consistent with the known existence of a different structure of the peptide in the membrane. However, the structure of the low pH translocation-competent state cannot be described, by the experimental criteria employed here, as unfolded. Therefore, either there is a change in average size that is too small to be detected or the structure change involves increased mobility of residue side chains with no change in mean size. The mobility change could allow increased accessibility into the peptide hydrophobic core of various probes and increased probability of contact of internal side chains with a membrane surface. Thus, the increased partition of the colicin channel peptide into nonionic detergent could result from an increased probability that the internal hydrophobic side chains can make contact with the detergent. Once this contact is made, either with the detergent or a membrane surface, a different unfolded conformation with exposed hydrophobic side chains would be stabilized.

ACKNOWLEDGMENTS

We thank S. Xu for experimental contributions to this work in its early stages, J. T. Bolin, W. J. Ray, Jr., K. Shirabe, and H. Y. Song for helpful discussions, I. Tessman for providing *E. coli* strain DM1187, Tim Walter for his contribution to the detergent partition experiment, Xiaoqing Yu for valued technical assistance, and Janet Hollister for careful and dedicated work on the manuscript.

Registry No. IAEDANS, 36930-63-9; Cl^-, 16887-00-6; colicin E1, 11032-88-5.

REFERENCES

Andrews, P. (1970) in *Methods of Biochemical Analysis* (Glick, D., Ed.) Vol. 18, pp 1–53, Interscience, New York.

Bishop, L. J., Cohen, F. S., Davidson, V. L., & Cramer, W. A. (1986) *J. Membr. Biol. 92*, 237–245.

Blewitt, M. G., Chung, L. A., & London, E. (1985) *Biochemistry 24*, 5458–5464.

Bordier, C. (1981) *J. Biol. Chem. 256*, 1604–1607.

Brunden, K. R., Uratani, Y., & Cramer, W. A. (1984) *J. Biol. Chem. 259*, 7682–7687.

Bullock, J. O., & Cohen, F. S. (1986) *Biochim. Biophys. Acta 856*, 101–108.

Bullock, J. O., Cohen, F. S., Dankert, J. R., & Cramer, W. A. (1983) *J. Biol. Chem. 258*, 9908–9912.

Chung, L. A., & London, E. (1988) *Biochemistry 27*, 1245–1253.

Cleveland, M. B., Slatin, S., Finkelstein, A., & Levinthal, C. (1983) *Proc. Natl. Acad. Sci. U.S.A. 80*, 3706–3710.

Davidson, V. L., Brunden, K. R., & Cramer, W. A. (1985) *Proc. Natl. Acad. Sci. U.S.A. 82*, 1386–1390.

Drapeau, G. R. (1976) *Methods in Enzymology* (Lorand, L., Ed.) Vol. 45, pp 469–475, Academic Press, New York.

Draper, R. K., & Simon, M. I. (1980) *J. Cell Biol. 87*, 849–854.

Eilers, M., & Schatz, G. (1986) *Nature 322*, 228–232.

Eilers M., & Schatz, G. (1988) *Cell 52*, 481–483.

Escuyer, V., Boquet, P., Perrin, D., Montecucco, C., & Mock, M. (1986) *J. Biol. Chem. 261*, 10891–10898.

Farabaksh, Z. T., & Wisnieski, B. J. (1989) *Biochemistry 28*, 580–585.

Gould, J. M., & Cramer, W. A. (1977) *J. Biol. Chem. 252*, 5491–5497.

Hudson, E. N., & Weber, S. (1973) *Biochemistry 12*, 4154–4160.

Kagan, B. L., Finkelstein, A., & Colombini, M. (1981) *Proc. Natl. Acad. Sci. U.S.A. 78*, 4950–4954.

Laemmli, U. K. (1970) *Nature (London) 227*, 680–685.

Laporte, D. C., Keller, C. H., Bradley, B. B., & Storin, D. R. (1981) *Biochemistry 20*, 3965–3972.

Lazdunski, C. J., Baty, D., Geli, V., Cavard, D., Morton, J., Llowbed, R., Howard, S. P., Knibiehler, M., Chartier, M., Varenne, S., Frenette, M., Dasseux, J. L., & Pattus, F. (1988) *Biochim. Biophys. Acta 947*, 445–464.

Montal, M., & Mueller, P. (1972) *Proc. Natl. Acad. Sci. U.S.A. 69*, 3561–3566.

Niles, W. D., Levis, R. A., & Cohen, F. S. (1988) *Biophys. J. 53*, 327–335.

Parker, M. W., Pattus, F., Tucker, A. D., & Tsernoglou, D. (1989) *Nature 337*, 93–96.

Partishall, K. H., Burchall, J. J., & Harvey, R. J. (1976) *J. Biol. Chem. 251*, 7011–7020.

Pattus, F., Cavard, D., Verger, R., Lazdunski, C., Rosenbusch, J., & Schindler, H. (1983) in *Physical Chemistry of Trans-Membrane Ion Motions* (Spoch, G., Ed) pp 407–413, Elsevier, New York.

Pattus, F., Heitz, F., Martinez, C., Provencher, S., & Lazdunski, C. (1985) *Eur. J. Biochem. 152*, 681–689.

Peterson, A. A., & Cramer, W. A. (1987) *J. Membr. Biol. 99*, 197–204.

Ramsay, G., Montgomery, D., Berger, D., & Freire, E. (1989) *Biochemistry 28*, 529–533.

Randall, L. L., & Hardy, S. J. S. (1986) *Cell 46*, 921–928.

Raymond, L., Slatin, S. L., Finkelstein, A., Liu, Q. R., & Levinthal, C. (1986) *J. Membr. Biol. 92*, 255–268.

Sandvig, K., & Olsnes, S. (1981) *J. Biol. Chem. 256*, 9068–9076.

Sandvig, K., & Brown, J. E. (1987) *Infect. Immun. 55*, 298–303.

Sandvig, K., & Moskaug, J. O. (1987) *Biochem. J. 245*, 899–901.

Schein, S. J., Kagan, B. J., & Finkelstein, A. (1978) *Nature (London) 276*, 159–163.

Shiver, J. W., Cramer, W. A., Cohen, F. S., Bishop, L. J., & de Jong, P. J. (1987) *J. Biol. Chem. 262*, 14273–14281.

Tessman, E. S., Gritzmacher, C. A., & Peterson, P. K. (1978) *J. Bacteriol. 135*, 29–38.

Vendittis, E. D., Polumbo, G., Parlato, G., & Bocchini, V. (1981) *Anal. Biochem. 115*, 278–286.

Wiley, D. C., & Skehel, J. J. (1987) *Annu. Rev. Biochem. 56*, 365–394.

Xu, S., Cramer, W. A., Peterson, A. A., Hermodson, M., & Montecucco, C. (1988) *Proc. Natl. Acad. Sci. U.S.A 85*, 7531–7535.

Yamada, M., Ebina, Y., Mayato, T., Nakazawa, T., & Nakazawa, A. (1982) *Proc. Natl. Acad. Sci. U.S.A. 79*, 2827–2831.