Colicin Ia Inserts into Negatively Charged Membranes at Low pH with a Tertiary but Little Secondary Structural Change†

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ABSTRACT: Colicin Ia, a member of the channel-forming family of colicins, inserts into model membranes in a pH- and lipid-dependent fashion. This insertion occurs with single-hit kinetics, requires negatively charged lipids in the target membrane, and increases in rate as the pH is reduced below 5.2. The low-pH requirement does not act by inducing a secondary structural change in colicin Ia, which remains 66% ● 4% α-helical between pHs 7.3 and 3.1 as determined by circular dichroism. The secondary structure also remains unchanged between pHs 7.3 and 4.2 in the hydrophobic environment provided by the detergent octyl β-D-glucopyranoside (β-OG). However, at pH 3.1 in the presence of β-OG, an 11% ± 3% decrease in the α-helical content is observed. Further, β-OG induces a change in tryptophan fluorescence and an altered pattern of proteolytic digestion, indicative of a tertiary structural change. This suggests that colicin Ia undergoes a tertiary but little or no secondary structural change in its transition from a soluble to a transmembrane protein.

The spontaneous insertion of proteins from aqueous environments into membranes is widely seen in biology. Examples include perforin (Podack et al., 1991), the C-9 component of the complement cascade (Stanley, 1989), several toxins which are pathogenic to humans such as diphtheria and cholera toxins (Holmgren, 1981; Kagan et al., 1981), and also members of the ion channel-forming family of colicins (Parker et al., 1990). The mechanism by which such molecules are able to insert into the hydrophobic environment of a lipid bilayer from aqueous solution is not fully understood at the molecular level. To understand both the biochemical and structural basis of this insertion process, we have focused on one member of this class of proteins, the bacteriocin colicin Ia.

Colicin Ia is a member of the ion channel-forming family of colicins, proteins that undergo a transition from a soluble to a transmembrane configuration. The members of this family, which includes colicins A, B, E1, Ia, Ib, K, and N, are synthesized in and released from *Escherichia coli* as soluble proteins. These colicins kill sensitive, target *E. coli* by first binding to a specific receptor on the outer membrane, translocating across the periplasmic space, and inserting into and forming ion-conducting channels within the inner membrane. The channels are relatively nonspecific and voltage-dependent and kill by depleting the target cell of its electrochemical potential [reviewed in Konisky (1982), Lazdunski et al. (1988), Cramer et al. (1990), and Pattus et al. (1990)].

Many of the proteins which spontaneously insert into membranes require low pH [reviewed in Parker et al. (1990)], and several also require negatively charged lipids (Shone et al., 1987; Cramer et al., 1990). Two of the channel-forming colicins, A and E1, require both low pH and negatively charged lipids (Bullock et al., 1983; Pattus et al., 1983; Davidson et al., 1984, 1985; Massotte et al., 1989) for membrane insertion. This insertion appears to be accompanied by a tertiary but little or no secondary structural change for both of these colicins (Brunden et al., 1984; Merrill et al., 1990; Lakey et al., 1991a; van der Goot et al., 1991).

Colicins A and E1 exist in vitro in at least two different membrane-associated states: a voltage-independent closed channel state and a voltage-dependent open channel state (Raymond et al., 1986; Slatin et al., 1986; Merrill & Cramer, 1990; Lakey et al., 1991a,b). The voltage-independent conformation is a membrane-associated intermediate between the water-soluble form and the transmembrane ion channel. While colicins do not conduct ions in this intermediate state, protease protection experiments indicate that regions of colicin E1 are closely associated with or inserted into the membrane (Xu et al., 1988).

We sought to determine whether colicin Ia has specific pH and lipid requirements for membrane insertion in the absence of a transmembrane voltage. We further examine the degree and type of structural change accompanying its transition from a hydrophilic to a hydrophobic environment. Our results are discussed in the context of the membrane insertion model proposed from the X-ray crystal structure of the soluble, channel-forming domain of colicin A (Parker et al., 1989).

MATERIALS AND METHODS

Purification of Colicin Ia. (A) Cell Growth and Lysis. Cells harboring the colicin Ia-producing plasmid pJK5 (Weaver et al., 1981) were grown to mid-log phase in LB culture medium (Miller, 1972) at 37 °C and induced by the addition of mitomycin C (Boehringer Mannheim) (stock solution of 2 mg/mL in 150 mM NaCl) to a final concentration of 0.2 μg/mL. Four to twelve hours after induction, the cell pellet was harvested by centrifugation at 5000 rpm for 10 min in a GSA or GSA3 rotor. The cell pellet was then solubilized in 1.5 mL of lysis buffer [50 mM tris(hydroxymethyl)-aminomethane (Tris),[1] pH 8, 2 mM EDTA, 0.1 mM DTT, 1 mM BME, 5% glycerol, and 100 mM NaCl] per gram of cells, and lysed using a probe sonicator, while the temperature was maintained below 10 °C. The lysate was diluted 4-fold

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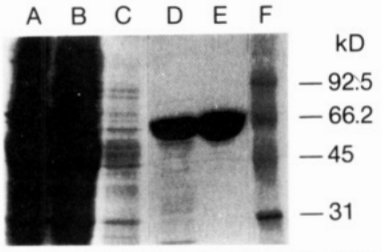

FIGURE 1: Purification of colicin Ia. Lane A, cell lysate; B, flow-through from the CM-Sephadex column, material that did not stick to the column upon loading; C, column wash, material that eluted from the CM-Sephadex column with a buffer wash; D, major OD_{280} peak eluted from the CM-Sephadex column with NaCl gradient; E, 13 μg of purified colicin Ia eluted from the HPLC column.

in buffer C (20 mM Tris, pH 8, 2 mM EDTA, 0.1 mM DTT, and 5% glycerol) containing 25 mM NaCl and centrifuged in an SS-34 rotor at 18 000 rpm for 20 min.

(*B*) *Ion-Exchange and Size-Exclusion Chromatography.* After centrifugation, the supernatant was applied to a CM-Sephadex C-50 column (Pharmacia) equilibrated with buffer C + 25 mM NaCl, at 4 °C. Colicin Ia adheres to the column matrix and is eluted as the major peak at OD_{280}, using a salt gradient which was 6× the column volume and increased linearly from buffer C + 25 mM NaCl to buffer C + 400 mM NaCl. This peak was concentrated at 4 °C (Amicon, YM30 filter) to 5–7 mg/mL and then applied to a Biosil TSK 250 HPLC column equilibrated with 50 mM NaCl and 20 mM citrate, pH 5.1 (buffer D). Colicin Ia elutes as a large, single peak after approximately 66 min, at a flow rate of 2 mL/min, and is sufficiently pure (Figure 1) to grow crystals which diffract to at least 2.8 Å (Ghosh, unpublished observation). Typically, 30 mg of purified protein per liter of cells was obtained. An extinction coefficient of 0.83 cm^2/mg was determined by measuring the OD_{280} (using a Shimadzu UV 160 spectrophotometer) of duplicate samples of colicin Ia. The mass of these samples was quantitated by amino acid content using a Beckman 121MB amino acid analyzer after gas-phase hydrolysis in 6 N HCl for 24 h at 108 °C.

Secondary Structure Determination Using Circular Dichroism. To assess secondary structure, circular dichroism spectra of colicin Ia were recorded on a Jasco 500 spectrophotometer, which was calibrated with ammonium *d*-camphor-10-sulfonate. As colicin Ia adheres to glass, all protein samples were handled using plastic tubes and plastic pipet tips. Colicin Ia (6.1 mg/mL stock solution in buffer D) was diluted to 0.1 mg/mL either with or without 1.5% β-OG (Pfanstiehl Lab, IL), in citrate/phosphate buffer, for which the pH was adjusted by titrating 5 mM citric acid with 10 mM dibasic sodium phosphate. Samples for circular dichroism were scanned from 240 to 190 nm in a 0.1-cm cell. To minimize colicin adherence to the quartz CD cell, it was washed with nitric acid and then rinsed extensively with water between each sample run. Duplicates of each sample were scanned 4 times and averaged, and a pH- and β-OG-adjusted base line was subtracted for each. Data were digitized on a Jasco DP-J500/PC data processor, with a sampling interval of 0.1 nm.

[1] Abbreviations: ANTS, 8-aminonaphthalene-1,2,3-trisulfonic acid; β-OG, octyl β-D-glucopyranoside; BME, 2-mercaptoethanol; CD, circular dichroism; CL, cardiolipin; DPX, *p*-xylylenebis(pyridinium bromide); DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; OD, optical density; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; TES, 2-[[tris(hydroxymethyl)methyl]amino]-ethanesulfonic acid; Tris, tris(hydroxymethyl)aminomethane.

Protein concentration was measured by OD_{280} and a modified Lowry assay (Markwell et al., 1978) for all samples. The CD spectra were normalized using the concentration that was determined by OD_{280}, with the exception of colicin Ia at pH 7.3 in the presence of 1.5% β-OG. In this sample, some aggregation was observed as determined by light scattering, so a modified Lowry assay alone was used to measure this protein concentration.

Secondary structure content was estimated by least-squares analysis, using a database of 15 solved protein structures (Chang et al., 1978; Yang et al., 1986). Standard deviations in the secondary structure estimation were determined by propagation of the standard deviation of CD measurements of duplicate samples with the standard deviation of protein concentration measurements.

Liposome Preparation. Vesicles were prepared following the reverse-phase evaporation (REV) method (Szoka & Papahadjopoulos, 1978). Approximately 10 μmol of either egg PC (Avanti Polar Lipids, Birmingham, AL) or purified (Kagawa & Racker, 1971) asolectin (Associated Concentrates, NY) was dried to a thin film and then rehydrated in 1 mL of diethyl ether and 1 mL of the following buffer: 12.5 mM ANTS (Molecular Probes, Eugene, OR), 45 mM DPX (Molecular Probes), 45 mM NaCl, 10 mM TES, and 0.1 mM EDTA. After 3 min of bath sonication, the emulsion was evaporated at room temperature for approximately 15 min to remove residual ether. To obtain a uniformly sized population, vesicles were extruded first through a 0.2-μm and then through a 0.1-μm polycarbonate membrane (Nucleopore) with a stream of argon at 40–50 psi. Unencapsulated ANTS and DPX were separated from the liposomes by applying the vesicles to a Sephadex G-75 column (1 × 20 cm) (Pharmacia) equilibrated with 10 mM TES, 0.1 mM EDTA, 140 mM NaCl, and 10 mM citrate (G-75 buffer). The osmolarity of this buffer was adjusted to that of the ANTS/DPX buffer with NaCl. The phospholipid content of the vesicles was measured by an inorganic phosphate analysis (McClare, 1971).

Monitoring Membrane Insertion by Fluorescence. Colicin Ia insertion into membranes was measured using an ANTS/DPX fluorescence leakage assay (Ellens et al., 1984). When coencapsulated inside liposomes, DPX quenches the fluorophore ANTS, resulting in no measurable fluorescence. The leakage of ANTS and DPX from vesicles into the surrounding medium leads to an increase in fluorescence, which results from the dequenching of ANTS. A solution of either asolectin or egg PC liposomes (50 μM final phospholipid concentration in G-75 buffer), adjusted to the desired pH with concentrated HCl, was stirred in a quartz cuvette at room temperature. The base-line fluorescence leakage (defined as 0% fluorescence leakage) from the vesicles was monitored for 100 s, at which time colicin Ia was added to a final molar lipid:protein ratio of 850:1 for the pH experiments, and at varying ratios for concentration dependence experiments. The final volume of each reaction mixture was 2 mL. Fluorescence release was recorded for 400 s; at 500 s, 50 μL of 10% Triton X-100 was added to each 2-mL sample, and samples were monitored for an additional 100 s. The addition of detergent leads to liposome lysis and defines 100% fluorescence leakage. All experiments were performed using a Spex Fluorolog-2 spectrofluorometer (Edison, NJ), with an excitation wavelength of 360 nm and an emission wavelength of 520 nm.

Proteolytic Digestion. Colicin Ia, both in the absence and in the presence of 1.5% β-OG, was digested with pepsin (Sigma). For all samples, a 6.1 mg/mL stock solution of colicin Ia in buffer D was diluted to 0.4 mg/mL in 50 mM

ammonium acetate buffer (pH 4.1), either with or without 1.5% β-OG. Immediately after the addition of pepsin (final concentration of 0.4 μM) to the solution of colicin Ia, 12 μL (from a total volume of 60 μL) was removed for the zero time point, added to sample buffer (Laemmli, 1970), and immediately frozen on dry ice to inhibit further proteolysis. At 5, 15, and 30 min after the addition of pepsin, additional 12-μL aliquots were removed and similarly treated. The digests were carried out both at room temperature and at 37 °C. Samples were examined by 16% SDS–PAGE (Laemmli, 1970).

Tryptophan Fluorescence. The tryptophan fluorescence of colicin Ia was measured both in the presence and in the absence of 1.5% β-OG, in citrate/phosphate buffer, pH 4.1 (buffer as described for circular dichroism experiments). Final sample concentrations were all 0.1 mg/mL, diluted from a 6.1 mg/mL stock solution in buffer D. All samples were prepared in plastic tubes, and plastic pipet tips were used for the transfer of any material due to problems of colicin Ia sticking to glass. Duplicate samples were excited at 278 nm and then scanned once from 300 to 400 nm with data recorded every 0.1 nm; data were corrected using the emission correction data file provided by Spex (MCORRECT). All experiments were performed on a Spex Fluorolog 1680 0.22m double-grating spectrometer (0.45-nm excitation bandpass and 4.5-nm emission bandpass) and the data were analyzed using Spex DM3000 software. A pH- and β-OG-adjusted base-line spectrum was subtracted from each sample.

RESULTS

Colicin Ia Inserts into Negatively Charged Lipids, but Only at Acidic pH. Colicin Ia spontaneously inserts into negatively charged asolectin membranes, with increasing efficiency as the pH is decreased below 5.2. Both the rate of fluorophore release and also the percent of total fluorophore leaked increase as the pH is lowered (Figure 2A,B). At pHs greater than 5.2, the addition of colicin Ia to negatively charged membranes results in no fluorophore release, indicating that membrane insertion does not occur at these pH values. Decreasing the pH is not sufficient for membrane insertion, however, as the addition of colicin Ia to vesicles made from the neutral lipid egg PC results in no fluorophore leakage over a pH range of 7.3–3.1. Thus, colicin Ia will insert into negatively charged phospholipids and not into neutral lipids, and will do so only at acidic pH.

The maximum rate of fluorophore leakage increases as the pH is decreased, with a midpoint ≤pH 4.5 (Figure 2B). The midpoint of the titration cannot be accurately defined, however, as below pH 4, the rate of fluorophore release increases dramatically upon colicin Ia addition to vesicles, but less than 50% of encapsulated fluorophore is released. This could be attributed to the decreased fluorescence of ANTS below pH 4 (Szoka, private communication), so these data were not included.

At pH 4.1, the maximum rate of fluorophore leakage from asolectin vesicles increases linearly with increasing colicin Ia concentration, within the range (12–177 nM) evaluated (Figure 3A,B). This supports a single-hit kinetic model of channel insertion (Wendt, 1970; Bruggemann & Kayalar, 1986; Levinthal et al., 1991), in which one molecule, or a rapidly associating molecular complex, inserts into the membrane to form a channel.

Colicin Ia releases 100% of encapsulated fluorophore at protein concentrations ≥89 nM (Figure 3A, curves a and b). The subsequent decrease to approximately 90% fluorophore leakage (Figure 3A, curves a and b) is likely due to light scattering resulting from vesicle aggregation. At 89 nM colicin

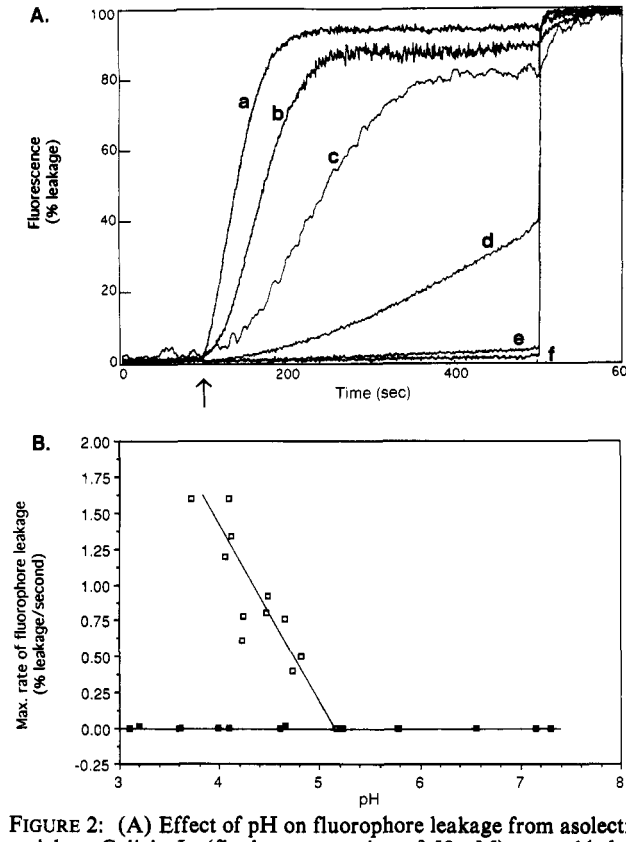

FIGURE 2: (A) Effect of pH on fluorophore leakage from asolectin vesicles. Colicin Ia (final concentration of 59 nM) was added to asolectin vesicles after 100 s of mixing (indicated with an arrow). Samples were stirred while the fluorescence leakage was monitored for an additional 5 min. (a) pH 4.1; (b) pH 4.5; (c) pH 4.6; (d) pH 4.8; (e) pH 5.2; (f) pH 5.7. 0 and 100% leakage values are defined under Materials and Methods. (B) Maximum rate of fluorophore leakage from asolectin (open symbols) and egg PC (closed symbols) vesicles as a function of pH. Rates were determined by measuring the slope at the steepest part of each leakage curve (units are percent leakage per second).

Ia, the protein to liposome ratio is estimated to be 140:1. This estimate assumes an average liposome diameter of 1000 Å (based on extrusion through a 1000-Å filter; see Materials and Methods) and a surface area of 70 Å^2/lipid (Small, 1986). Assuming colicin Ia acts as a monomer, a Poisson distribution shows that at 89 nM colicin Ia, the probability of finding a liposome with no protein molecules attached is diminishingly small, in essence zero. Since all colicin Ia is associated with liposomes as determined by ultracentrifugation (data not shown), multiple colicin Ia molecules will be attached to each vesicle at this protein concentration. Thus, as 100% of the fluorophore is released only at an estimated colicin:liposome ratio of 140:1, on the order of 1% of colicin Ia molecules are functionally inserting under these experimental conditions.

The Transition from a Hydrophilic to a Hydrophobic Environment Requires Little or No Change in Secondary Structure. To determine whether the acidic pH required for membrane insertion induces a structural change in colicin Ia, the secondary structure composition of the soluble form of colicin Ia was evaluated at pHs between 7.3 and 3.1, using circular dichroism (CD). A comparison of spectra at four pH values reveals that the overall shapes and zero crossings (wavelength at which molar ellipticity $\epsilon = 0$) are essentially identical but that the amplitudes of these spectra are somewhat different (Figure 4A). As the shape and zero crossing of a CD spectrum directly reflect the proportional contribution of each secondary structure element in a protein (α-helix, β-sheet,

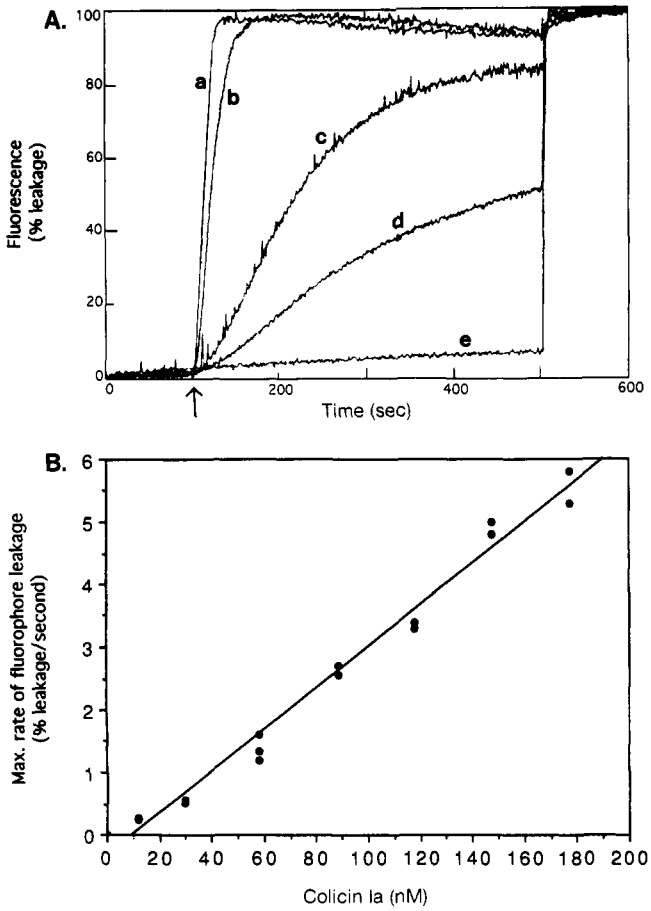

FIGURE 3: (A) Rate of fluorescence leakage from asolectin vesicles at pH 4.1 as a function of colicin Ia concentration. Colicin Ia was added after 100 s of mixing (indicated with an arrow) at the following concentrations: (a) 177; (b) 89; (c) 30; (d) 12; (e) 0 nM. (B) Maximum rate of fluorophore leakage from asolectin vesicles as a function of colicin Ia concentration. Leakage rates determined as in the legend for Figure 1B.

turn, random coil), any genuine change in secondary structure would lead to a change in either the shape or the zero crossing, or both, of that spectrum. The overall amplitude of a raw spectrum, however, is directly proportional to protein concentration.

To determine the source of the differences among the spectra of colicin Ia at the different pH values, the CD spectra were linearly scaled to optimize overlap (Figure 4B). After the spectra were scaled, the calculated secondary structure proportions were identical to within ±1%, indicating that the proportion of α-helix to random coil in colicin Ia does not change over the pH range 7.3–3.1. Within this pH range, the percent α-helix remained constant at 66% ± 4%, and the remaining secondary structure was comprised of random coil (34% ± 4%) (Table I). Scaling the curves supposes that there is variation in concentration between samples, possibly due to colicin Ia sticking to glass.

To test whether a hydrophobic environment alone or in combination with pH induces a change in secondary structure content, CD spectra of colicin Ia solubilized in the nonionic detergent β-OG were evaluated across a pH range of 7.3–3.1 (Figure 4C). β-OG was used to mimic the hydrophobic nature of the membrane in order to minimize problems due to light scattering from lipids (Wallace & Mao, 1984). The secondary structure content of colicin Ia at pHs of 7.3 and 4.1 is constant at 68 ± 1% α-helix and 32 ± 1% random coil, in the presence of 1.5% β-OG (critical micelle concentration = 0.8%) (Table I). At pH 3.1 in 1.5% β-OG, however, the amount of α-helix drops to 59.8 ± 1%, with a compensatory increase in the percent of random coil. Notably, the zero crossing is shifted to a lower wavelength by 0.8 nm (Figure 4C, inset, as compared to Figure 4A,B, insets). This reflects a decrease of 11 ± 3% in the amount of α-helix, as reflected in both the overall shape and zero crossing of this spectrum, when compared with the spectra of colicin Ia in β-OG at pHs 4.2 and 7.3.

Therefore, at pH 3.1, the structure of colicin Ia is slightly altered by the presence of a hydrophobic environment. Between pH 7.3 and pH 4.2, however, neither a hydrophobic environment nor an acidic pH significantly alters the amounts of secondary structure of this highly α-helical protein. As colicin Ia will release the contents of liposomes below pH 5.2, membrane insertion does not require a significant change in secondary structure content.

The Transition from a Hydrophilic to a Hydrophobic Environment Is Accompanied by a Tertiary Structural Change. To assess tertiary structural changes as a function of the hydrophobicity of the environment, proteolytic digestion of colicin Ia was carried out in the presence and absence of β-OG at pH 4.1. The susceptibility of colicin Ia to proteolysis is altered in the presence of 1.5% β-OG. After a 30-min digestion at 37 °C in the absence of 1.5% β-OG, a strong proteolytic product of ~25 kDa remains uncleaved (Figure 5A, lanes 2–4). This 25-kDa product is not visible in a parallel digest in the presence of 1.5% β-OG, indicating an increased susceptibility to proteolysis in the presence of this detergent (Figure 5A, lanes 6–8). The different digestion pattern does not result from a temperature-induced secondary structural change of colicin Ia in the presence of detergent, as the α-helical content of colicin Ia in 1.5% β-OG at 25 °C is identical to that at 37 °C (data not shown). A similar digestion carried out at 25 °C reveals that the reaction products from colicin Ia digested in the presence of 1.5% β-OG are different from those of colicin Ia digested in the absence of detergent (Figure 5B, lanes 1–8).

The appearance of different proteolytic digestion products in the presence of detergent can be explained in one of two ways. Either colicin Ia has undergone a tertiary structural change, resulting in the appearance of new proteolytic cut sites and the disappearance of others, or the protein structure remains unaltered in detergent but the presence of β-OG limits the access of pepsin to existing cut sites. That the digestion of colicin Ia proceeds more rapidly in the presence of detergent (Figure 5A) argues in favor of a structural change in the protein, rather than a masking of available cut sites by β-OG.

Further evidence supporting a tertiary structural change in the presence of a hydrophobic environment comes from the tryptophan fluorescence of colicin Ia. The addition of 1.5% β-OG to colicin Ia leads to a 15% increase in fluorescence intensity at both pH 4.2 and pH 7.3, as well as a small shift of 1 nm in the wavelength maximum (from 321 to 322 nm at pH 4.2 and from 323 to 324 nm at pH 7.3) (Figure 6), indicating that the detergent alters the environment of tryptophan residues. When scaled to correct for protein absorption to glass, these tryptophan fluorescence spectra (Figure 6) were not superimposable, indicating a genuine change in tryptophan environment in the presence of detergent, further consistent with a tertiary structural change in colicin Ia.

In contrast, pH alone has a minor effect on the environment surrounding tryptophan residues. In the absence of β-OG, the tryptophan fluorescence spectrum of colicin Ia at pH 7.3 exhibits a maximum near 323 nm (Figure 6). Lowering the pH to 4.2 results in a very slight blue shift of 1–2 nm to 321

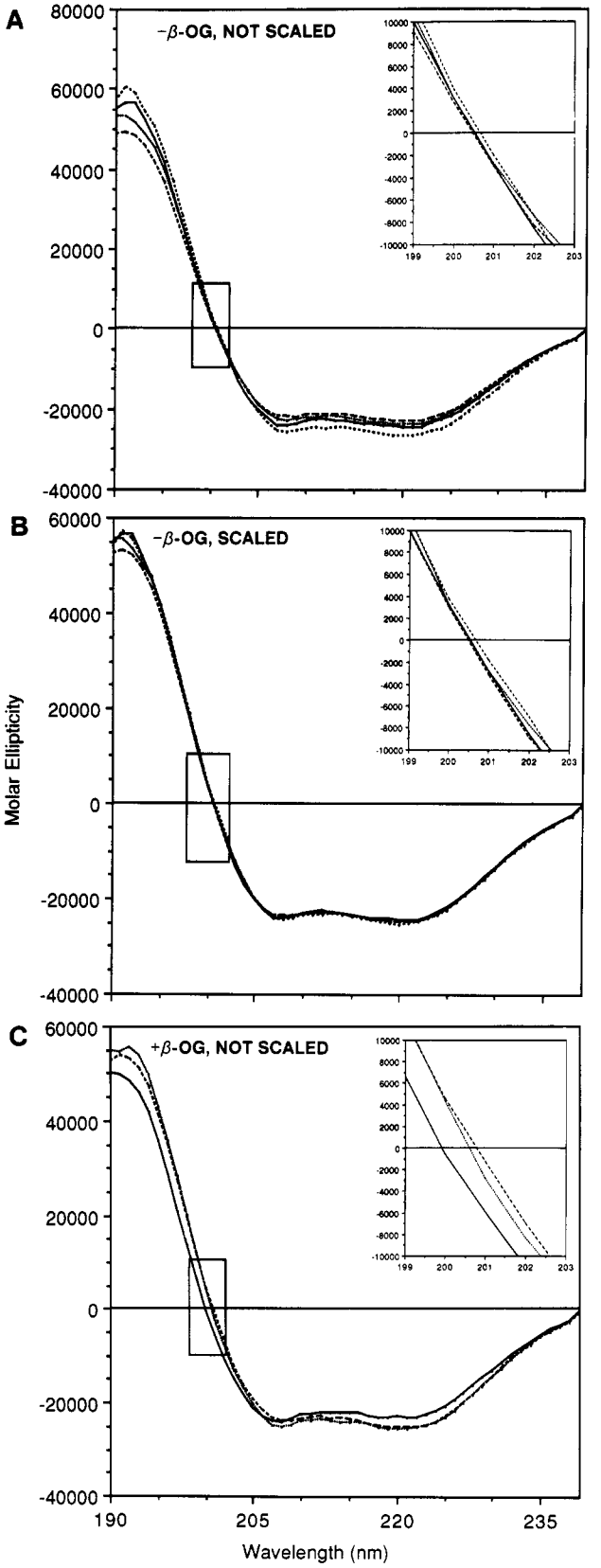

FIGURE 4: Circular dichroism of colicin Ia as a function of pH and 1.5% β-OG. Boxed areas on spectra are displayed as insets. (A) CD spectra in the absence of β-OG, data not scaled; (B) CD spectra in the absence of β-OG, data scaled; (C) CD spectra in the presence of β-OG, data not scaled. Units of molar ellipticity are degrees centimeter square per decimole. pH 3.1, solid line; pH 4.2, dotted line; pH 5.1, shorter dashed line; pH 7.3, longer dashed line.

nm, with a 2% change in fluorescence intensity. These small changes in both the wavelength maximum and the fluorescence intensity indicate little or no significant change in the protein tertiary structure in response to pH changes alone.

Table I: Percent α-Helix of Colicin Ia ± β-OG[a]

	−β-OG		+β-OG	
pH	calculated	scaled	calculated	scaled
3.1	66.5	66.5	59.8	NA[c]
4.2	64.5	66.5	68.5	NA
5.1	70.2	67.5	ND[b]	NA
7.3	61.3	65	68.3	NA

[a] In all cases, the non-α-helical secondary structure is random coil, with no calculated β-sheet. Standard deviations for all reported values are less than ±2%. Scaling of values is described under Results. [b] Not determined. [c] Not applicable.

DISCUSSION

Colicin Ia is a member of a class of proteins which spontaneously insert into membranes from aqueous environments. For colicin Ia, insertion will occur only in the presence of negatively charged membranes and only at low pH. The dependence on negatively charged lipids may be indicative of an electrostatic attraction between colicin Ia and its target membrane surface. The inner *E. coli* membrane, into which colicin Ia inserts and forms a channel in vivo, has a phospholipid composition of PE/PG/CL in a ratio of 74:19:3 (Gennis, 1989), which results in an overall net negative charge of 0.25 electron per lipid. The requirement for negatively charged lipids suggests an electrostatic attraction between the membrane and positively charged colicin Ia, which has a calculated isoelectric point of 9.72 (Stroud, PREDICT program). The mechanism of electrostatic attraction is probably conserved across the channel-forming colicin family, as both colicins A and E1 are also basic proteins (isoelectric points of 8.86 and 10.08, respectively) and require negatively charged phospholipids for membrane insertion (Bullock et al., 1983; Massotte et al., 1989).

Specific residues that might be involved in this electrostatic interaction have not yet been directly identified for colicin Ia. However, it has been proposed on the basis of the X-ray crystal structure of the colicin A channel-forming fragment that eight positively charged residues are responsible for the interaction of that protein with a negatively charged target membrane (Parker et al., 1989). Of these eight residues, only two positive charges are conserved between colicin A and colicin Ia. The overall number of positive charges within the channel-forming domains, however, is similar between these 2 colicins: there are 28 positively charged residues in the colicin A channel-forming domain and 30 in the same region of colicin Ia. Therefore, although the general mechanism of electrostatic attraction is likely to be similar between colicin A and colicin Ia, the details of this mechanism may differ.

In common with the other channel-forming colicins, colicin Ia also requires a low pH for membrane insertion (Bullock et al., 1983; Pattus et al., 1983; Davidson et al., 1984, 1985). This requirement for low pH probably reflects the necessity to protonate colicin Ia, phospholipid head groups, or both. Since the midpoint of the maximum rate of fluorophore leakage is ≤pH 4.5 (Figure 2B), it is likely that the titration of groups with pK's near this value is responsible for this effect. The secondary phosphate groups in asolectin, which are strongly acidic with a pK < 2, would not be significantly titrated between pH 4 and 5. While the phosphatidic acid groups in asolectin have a pK near pH 4 and thus would be titrated between pH 4 and 5, they constitute only 8% of the asolectin mixture. It is more likely that low pH is required for protonation of

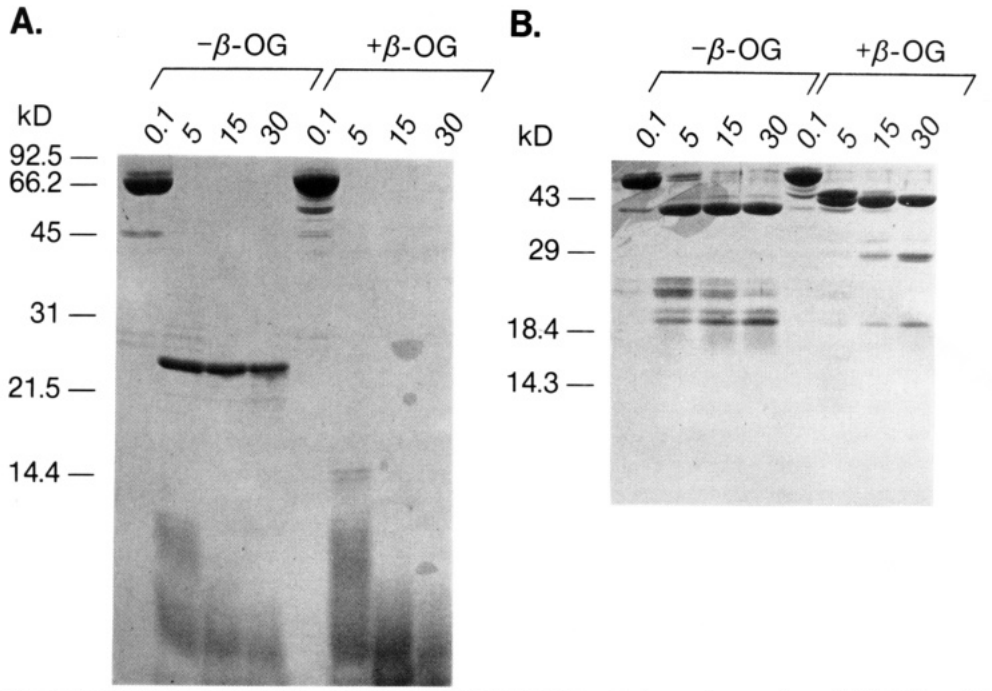

FIGURE 5: Pepsin digest of colicin Ia ± 1.5% β-OG. (A) Digest at 37 °C; (B) digest at room temperature. 0.1, 5, 15, and 30 refer to minutes of digestion. (One large and three smaller bubbles are visible between lanes 7 and 8, panel A.) 0.1 time point was taken immediately after pepsin addition.

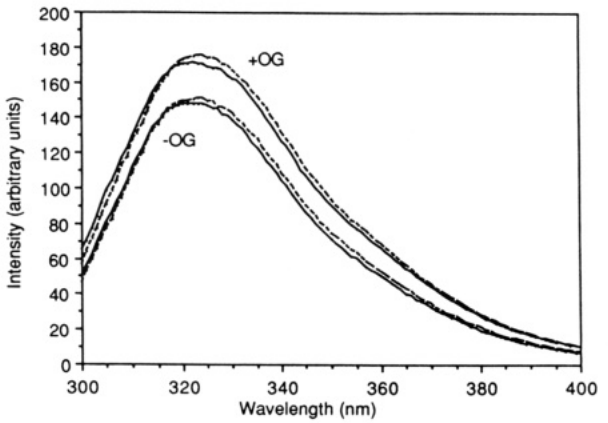

FIGURE 6: Tryptophan fluorescence spectra of colicin Ia in the presence and absence of 1.5% β-OG. Solid line, pH 4.1; dashed line, pH 7.3.

negatively charged carboxyl groups on aspartic and glutamic acids, whose p*K* values are near pH 4.5. The protonation of the carboxyls may serve to reduce the repulsion between the negatively charged membrane and these negatively charged groups on colicin Ia. The protonation might further serve to neutralize carboxyls that are to cross the membrane. Site-directed mutagenesis experiments on the channel-forming domain of colicin E1 indicate that more than one carboxylic acid residue must be protonated to account for the pH-dependent increase in channel-forming activity (Davidson et al., 1985; Shiver et al., 1987, 1988).

As the insertion of colicin Ia into asolectin vesicles is pH-dependent, we sought to understand whether lowering the pH leads to a conformational change in the protein. CD spectra indicate that soluble colicin Ia remains highly α-helical (66%) as the pH is decreased from 7.3 to 4.2, reflecting no change in secondary structure within this range. Furthermore, lowering the pH from 7.3 to 4.2 induces little or no tertiary structural change, as determined by tryptophan fluorescence. Similarly, the tertiary structure of the colicin A channel-forming domain does not change as the pH is varied between 7 and 4, and the channel-forming regions of both colicins A and E1 exhibit little or no change in secondary structure as the pH is lowered to 4 (Brunden et al., 1984; van der Goot et al., 1991).

Below pH 4, however, a pH-induced structural change is seen in both colicins A and E1. Spectroscopic analysis of colicin A demonstrates the appearance of a "molten-globule" form of the protein, in which there is a compact, water-excluding protein core and significant secondary structure, but no native tertiary structure (van der Goot et al., 1991). A tertiary structural change has also been identified for colicin E1, as determined by altered proteolytic digestion patterns of the protein and increased accessibility below pH 4 to a fluorescent probe on a previously buried cysteine (Merrill et al., 1990). For colicin Ia, the secondary structure of the soluble form below pH 4 is not altered.

In contrast to the effect of pH alone, the presence of detergent at pH 3.1 leads to an 11% decrease in the α-helical content of colicin Ia. In addition to a loss of α-helix, the presence of detergent may also lead to a rearrangement of tertiary structure. At pH 4.1, proteolytic digestions of colicin Ia in the presence and absence of β-OG reveal different products, consistent with the appearance of new proteolytic cut sites and loss of others, indicating a possible change in tertiary structure. Furthermore, at both pHs 7.3 and 4.2, the addition of 1.5% β-OG leads to an increase in tryptophan fluorescence intensity, indicating a change in the environment surrounding tryptophan residues, also consistent with a tertiary structural change in the protein. This type of structural rearrangement appears to be conserved within the colicin family, as colicin A also exhibits a change in tertiary structure upon exposure to a membrane (Lakey et al., 1991a).

Exposure to a hydrophobic environment evokes no change in the secondary structure of colicin Ia at either pH 7.3 or pH 4.2. Similarly, colicins A and E1 undergo little or no secondary structural change within this pH range in the presence of either lipids or detergent (Brunden et al., 1984; Goormaghtigh et al., 1991; Rath et al., 1991). It therefore appears that a tertiary but not secondary structural change accompanies the

membrane insertion of members of this colicin family.

Such a change is probably necessary for this family of colicins to insert into membranes and form ion channels. Colicins A, E1, and Ia are all highly α-helical proteins (Brunden et al., 1984; Pattus et al., 1985; Parker et al., 1989; Wormald et al., 1990), and although they have very little sequence identity, they share a strong amphipathic α-helical characteristic within their carboxy-terminal one-third which comprises the channel-forming region [Dankert et al., 1982; Ohno-Iwashita & Imahori, 1982; Martinez et al., 1983; Cavard et al., 1986; Shiver et al., 1989; and reviewed in Baty et al. (1988)]. The tertiary structural changes probably reflect an alteration in these amphipathic α-helices from a soluble conformation in which the apolar faces are packed within the protein interior to a membrane configuration in which the apolar faces are exposed to the lipid (Parker et al., 1989; Lakey et al., 1991b). A two-step model explaing this membrane-insertion process has recently been proposed in which colicins, after first binding to a membrane in an "umbrella" conformation, are then driven into the bilayer by a transmembrane voltage (Parker et al., 1989).

The "umbrella" model provides an explanation for the finding that not all fluorophore is released from vesicles in the ANTS/DPX leakage assay, even in the presence of multiple copies of colicin Ia per vesicle. In this model, colicin molecules first become anchored within a target membrane via a hydrophobic helical hairpin, but insert fully only after the application of a transmembrane voltage. We have found that in the absence of a transmembrane voltage, only $\sim$1 in approximately 140 colicin Ia molecules will spontaneously insert into the membrane and form channels. For colicin E1, the presence of a transmembrane voltage leads to increased channel formation in liposomes (Merrill & Cramer, 1990). The high colicin to liposome ratios necessary for fluorophore release in our case could also be explained by the possibility that colicin Ia forms a channel as an oligomer. It seems more likely, however, that the lack of a transmembrane voltage is responsible for this effect, since most recent evidence argues in favor of the pore-forming colicins acting as monomers (Bruggemann & Kayalar, 1986; Peterson & Cramer, 1987; Slatin, 1988; Levinthal et al., 1991).

The membrane insertion of colicin Ia appears to require a change in tertiary structure without a significant change in secondary structure. A similar structural alteration has been observed for filamentous bacteriophage Pf1 coat protein. It also undergoes a tertiary structural change with no corresponding change in secondary structure during membrane-mediated viral assembly, as determined by neutron diffraction and nuclear magnetic resonance (NMR) studies (Nambudripad et al., 1991). The tertiary alteration of preexisting secondary structures may represent a generalized mechanism of action for proteins which exist in both hydrophobic and hydrophilic environments. The study of the channel-forming family of colicins will greatly aid in understanding the mechanism of insertion as well as the structural basis for the transition from a hydrophilic to a hydrophobic environment.

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