Acidic Interaction of the Colicin A Pore-Forming Domain with Model Membranes of *Escherichia coli* Lipids Results in a Large Pertubation of Acyl Chain Order and Stabilization of the Bilayer†

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ABSTRACT: ^2H and ^{31}P NMR techniques were used to study the effects on acyl chain order and lipid organization of the well-characterized pore-forming domain of colicin A (20-kDa thermolytic fragment of colicin A) upon insertion in model membrane systems derived from the *Escherichia coli* fatty acid auxotrophic strain K 1059, which was grown in the presence of [11,11-$^2H_2$]-labeled oleic acid. Addition of the protein to dispersions of the *E. coli* total lipid extract, in a 1/70 molar ratio of peptide to lipids, resulted in a large pH-dependent decrease in quadrupolar splitting of the ^2H NMR spectra. The decrease of the quadrupolar splitting obtained at the various pH values was correlated with the pH dependence of the insertion of the protein in monolayer films using the same *E. coli* lipid extracts. The p*K* governing the perturbing effects on the order of the fatty acyl chains was around 5, in agreement with the values of the pH-dependent conformational changes of the pore-forming domain of colicin A required for membrane insertion as reported by van der Goot et al. [(1991) *Nature 354*, 408–410]. ^{31}P NMR measurements show that the bilayer organization remains intact upon addition of the protein to dispersions of lipid extract. Surprisingly, ^{31}P NMR measurements as a function of temperature indicate that the pore-forming domain of colicin A even stabilizes bilayer lipid structure at pH 4. Both the large effect of the protein on acyl chain order and its bilayer-stabilizing activity are indicative of a surface localization of the protein. Implications of these data for the membrane insertion of the colicin A pore-forming domain are discussed.

Pore-forming colicins are bactericidal proteins able to form voltage-dependent channels in *Escherichia coli* cytoplasmic membranes. They form well-defined voltage-gated ion channels in artificial membranes (Schein et al., 1978). Like many toxins, colicins are organized in structural domains, each domain carrying one function associated with the toxin's lethal activity (Baty et al., 1988). The central domain is responsible for the binding to a receptor on the bacterial surface, the N-terminal domain is involved in the translocation step across the outer membrane, and the C-terminal domain carries the ionophoric activity.

The C-terminal fragments of pore-forming colicins can be purified after cleavage of the entire molecule by proteases (Martinez et al., 1983; Bullock et al., 1983). These pore-forming domains are water-soluble and possess ionophoric activities similar to that of the intact protein when lipid planar bilayers or liposome systems are used.

In vitro, the channel formation can be divided into two distinct steps: a high-affinity lipid binding site spontaneously inserts into the lipid bilayer and then the application of a trans-negative membrane potential causes insertion of additional helices of the molecule and channel opening [for reviews see Slatin (1988), Cramer et al. (1990), and Pattus et al. (1990)].

The initial insertion of the C-terminal fragments in lipid vesicles, planar bilayers, or monolayer films requires acidic pH and negatively charged phospholipids (Pattus et al., 1983; Raymond et al., 1985; Davidson et al., 1984; Nogeuira et al., 1988; Frenette et al., 1989; Massotte et al., 1989). At neutral pH, pore-forming domains possess weak affinity for lipids. Protonation of one or more amino acids is required for membrane binding (Pattus et al., 1983; Davidson et al., 1985; Shiver et al., 1987). For the thermolytic fragment of colicin E1, which is highly homologous to the colicin A pore-forming domain, the attainment of a competent state for membrane insertion at acidic pH was accompanied by an increased accessibility/sensitivity to proteases and peptide rearrangement (Merrill et al., 1990). Solution NMR studies on the same peptide suggest that conformational changes required for membrane insertion involve reduction of local stability of parts of the solution structure rather than creation of a new unfolded state (Wormald et al., 1990). Kinetic studies on the membrane insertion at low pH of the pore-forming domain of colicin A showed that the intermediate for membrane insertion was a molten globular conformation (van der Goot et al., 1991).

The crystal structure of the pore-forming domain of colicin A has been refined at 2.4-Å resolution (Parker et al., 1989, 1992). The polypeptide chain consists of 10 α-helices with a hydrophobic core made of a helical hairpin (helices 8 and 9) which is buried in the soluble form of the protein. Based on the colicin A structure, a model for membrane insertion was proposed (Parker et al., 1989). The initial binding of the protein to the membrane was proposed to be an electrostatic interaction between a ring of positive charges located in the same plane and negatively charged phospholipids (Parker et al., 1989). Helices 8 and 9 have been hypothesized to be inserted into the membrane bilayer in the first potential-

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independent step leaving other helices at the membrane surface ("umbrella model") (Parker et al., 1989). Spectroscopic studies on the environment of the aromatic residue in the soluble and membrane-bound forms support the reorientation of the helices proposed in the model in going from the water-soluble to the membrane form (Lakey et al., 1991a,b). While changes in the tertiary structure occur during membrane insertion, the elements of the secondary structure are conserved (Brunden et al., 1984; Pattus et al., 1985; Lakey et al., 1991a,b).

In the presence of a potential, voltage-sensitive fragments insert into the membrane (Slatin et al., 1986; Jakes et al., 1990; Merrill & Cramer, 1990). The molecularity and the structure of the channel itself are still controversial questions (Bruggeman & Kayalar, 1986; Petersen & Cramer, 1987; Slatin, 1988; Pattus et al., 1990; Parker et al., 1992).

Despite the increasing amounts of data on the structural changes of the pore-forming domain of colicins when they interact with lipids, no information is available about the effects of the toxin on the lipids themselves. In this study, using ^{2}H NMR[1] and ^{31}P NMR techniques, we investigate the effects of the colicin A pore-forming domain on lipid acyl chain order and on membrane lipid organization of model membrane systems derived from *E. coli*. For this purpose we used a fatty acid auxotrophic mutant of *E. coli*, which was grown in the presence of deuterium-labeled oleic acid. The localization of the ^{2}H label was chosen at the 11-position next to the carbon double bond, because at this position the deuterons in the lipids are motionally equivalent and give rise to a relatively small quadrupolar splitting ($\Delta\nu_q$) (Chupin et al., 1986), thereby optimizing the signal-to-noise ratio in the ^{2}H NMR measurements. Previously, phospholipids carrying this label have been successfully employed to study the interaction of several peptides and proteins with lipid bilayers (Jordi et al., 1990; Chupin et al., 1987; De Kroon et al., 1991). More recently, the phase behavior and lipid acyl chain order of *E. coli* derived phospholipids, similar to those used in this study, were characterized (Killian et al., 1992). We will show by ^{2}H and ^{31}P NMR that the membrane insertion of the colicin A pore-forming domain triggered at acidic pH results in large perturbing effects of acyl chain order while the bilayer organization is maintained and even stabilized. The pH dependence of this process is correlated to that of insertion of the protein into monolayers of the *E. coli* lipids. The results provide new insights into the potential-independent insertion of pore-forming colicins.

MATERIALS AND METHODS

Deuterated oleic acid ([11,11-$^2H_2$]oleic acid) was synthesized as described by Farren et al. (1984) with modifications according to Chupin et al. (1986). Deuterium-depleted water was obtained from Sigma (St. Louis, MO). All other chemicals were of analytical grade.

Preparation of the Colicin A Thermolytic Fragment. Preparation of the colicin A thermolytic fragment was adapted from Cavard and Lazdunski (1979). A 10-L culture of C600 (pColA9) strain (Lloubés et al., 1986) was grown in LB (Luria broth) medium to an OD_{600nm} of 2 and induced for 2 h with 0.125 μg/mL mitomycin C. Cells were harvested and the pellet was resuspended with a Waring blender in 80 mL of extraction buffer [700 mM sodium chloride, 10 mM phosphate buffer (pH 6.8), 1 mM ethylenediaminetetracetate (EDTA), and 0.2 mM phenylmethanesulfonyl fluoride (PMSF)]. The suspension was sonicated in ice-water 3 times for 30 s and treated with DNase (10 μg/mL) in the presence of 2 mM magnesium chloride for 20 min on ice. The suspension was centrifuged at 16000*g* for 1 h in a Sorvall GSA rotor. The extraction was repeated on the pellet. Supernatants were pooled and precipitated with ammonium sulfate (430 g/L) and centrifuged at 15000*g* for 30 min in a Sorvall SS34 rotor. The pellet was resuspended in 100 mL of buffer 1 (10 mM phosphate buffer, pH 6.8, 1 mM EDTA, and 0.2 mM PMSF). The suspension was dialyzed against 12 L of buffer 1. The dialyzed supernatant was clarified and loaded on a 50-mL column of a S-Fastflow anionic exchanger (Pharmacia) equilibrated with buffer 1. The column was developed at a flow rate of 1.5 mL/min with a 0–0.4 M sodium chloride linear gradient. The protein eluted at 100 mM sodium chloride. Fractions were pooled, concentrated with Amicon PM10 membranes, and dialyzed against 12 L of buffer 2 (20 mM morpholinoethanesulfonic acid (MES), pH 5.5, and 1 mM EDTA). The dialyzed supernatant was clarified and loaded on an 8-mL S-Fastflow anionic exchanger (Pharmacia) equilibrated with buffer 2 and eluted as mentioned above. Around 350 units (OD_{280nm}) of colicin was obtained. Fractions were precipitated with ammonium sulfate (561 g/L) and dialyzed against 12 L of buffer 3 (20 mM Tris-HCl, pH 7.8). The dialyzed supernatant was clarified and treated for 16 h at 37 °C with 1% thermolysin in 20 mM Tris-HCl, pH 7.8, 2 mM sodium acetate, and 6 mM β-mercaptoethanol. Efficiency of the digestion was monitored on SDS–PAGE. The treated material [2 mL; 50 units (OD_{280nm})] was loaded on a Superose 12 HR16/50 column equilibrated with buffer 3. The flow rate was 1 mL/min. The elution volume of the thermolytic peptide was 60 mL. Fractions (110 units at OD_{280nm} in 37 mL) were dialyzed against 50 mM ammonium bicarbonate. The dialyzed fractions were aliquoted in fractions of 5.5 mg of thermolytic peptide. The concentration of the peptide was checked by absorbance at 280 nm using as molar extinction coefficient the value ($E_{280} = 2.43 \times 10^{-4}$ M^{-1} cm^{-1}) reported by Massotte et al. (1989). Fractions were then lyophilized.

Preparation of Total Lipid Extracts. Phospholipid extracts were prepared of cells of *E. coli* strain K1059 (Overath et al., 1970), which was a kind gift of Dr. P. Overath. The cells were grown at 37 °C in Cohen–Rickenberg (CR) mineral salts medium (Ankaru, 1967), supplemented with 0.5% (v/v) glycerol, 0.3% (w/v) casamino acids, 0.2% (v/v) Brij-35, and 0.01% (v/v) [11,11-$^2H_2$]oleic acid. Cells were grown till the late logarithmic phase, after which they were chilled on ice and harvested by low-speed centrifugation. Total phospholipid extracts were obtained by extraction according to Bligh and Dyer (1959) and purified by chromatography on a silica (Polygosil 63–100 μm) column as described (Killian et al., 1992). The phospholipid composition (mole percent) of the extract is 79% phosphatidylethanolamine, 15% phosphatidylglycerol, and 6% cardiolipin, and the fatty acid composition is 14:0 (7.1%), 16:0 (34.2%), 16:1 (1.7%), 18:0 (2.9%), 18:1 (51%), and 18:2 (3.1%) (Killian et al., 1992).

For preparation of NMR samples, a dry film of 20 μmol of phospholipid was hydrated with 0.5 mL of a deuterium-depleted buffer containing 50 mM sodium chloride and 100 mM trisodium citrate at various pH values in the range of 4–6. The samples were incubated for 1 h at 30 °C, after

[1] Abbreviations: ^{2}H NMR, deuterium nuclear magnetic resonance; ^{31}P NMR, phosphorus nuclear magnetic resonance; DOPG, dioleoylphosphatidylglycerol; Tris, tris(hydroxymethyl)aminomethane; $\Delta\nu_q$, quadrupolar splitting; LB, Luria broth; EDTA, ethylenediaminetetraacetate; PMSF, phenylmethanesulfonyl fluoride; MES, morpholinoethanesulfonic acid; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; CR, Cohen–Rickenberg mineral salts medium.

which they were gently vortexed and subjected to several cycles of freeze–thawing.

Addition of Colicin A. Lyophilized thermolytic peptide (5.5 mg) was dissolved in 0.5 mL of deuterium-depleted buffer containing 50 mM sodium chloride and 100 mM sodium citrate at various pH values between 4 and 6. The concentration of the solution was measured by absorbance at 280 nm and adjusted to 10 mg/mL. To obtain the desired final molar ratio, an aliquot of the peptide solution was added to 500 μL of hydrated phospholipids (20 μmol) at the same pH. After gentle mixing, the final volumes were adjusted to 1 mL using the appropriate buffer. The pH of the solutions did not change upon dissolving the peptide or after adding the protein to the hydrated phospholipids.

NMR Measurements. ^{2}H NMR spectra were recorded at 46.1 MHz on a Bruker MSL 300 spectrometer using a quadrupolar echo pulse sequence (Davis et al., 1976) with a 21-μs 90° pulse, a 2K time domain, an echo delay of 35 μs, and a 100-ms delay between pulses. The spectral width was 71.5 kHz and typically 10 000–50 000 scans were accumulated. The signal-to-noise ratio was increased by applying an exponential multiplication prior to Fourier transformation, resulting in a 200-Hz line broadening. All ^{2}H NMR spectra were recorded at 30 °C.

^{31}P NMR spectra were recorded at 121.4 MHz on the same spectrometer, using a 12-μs 90° pulse, a 38.5-kHz spectral width, 4K data points, and a 1-s interpulse time. A gated decoupling program was used with an input power of 3 W during 2.6% of the interpulse time. Three thousand free induction decays were accumulated and an exponential multiplication was applied, resulting in 100-Hz line broadening. All spectra shown are normalized to the same height. Percentages of "isotropic" and "bilayer" component were calculated from the integrated areas of the spectra before and after computer substraction of the putative pure components, with an estimated error of about 10%.

Monolayer Measurements. Interfacial measurements were performed at 22 °C in a thermostatically controlled box (Demel, 1982) with a 50 mM sodium chloride and 100 mM sodium citrate buffer as a subphase and pH values as indicated. The monolayers of *E. coli* total lipid extract were spread from chloroform/methanol (8/2 v/v) solution to give an initial surface pressure of 30 mN/m. The surface pressure was measured by the Wilhelmy method. The Teflon dish had a volume of 5.0 mL and a surface area of 4.9 cm^2. The colicin A pore-forming domain was added to the subphase from a 1 mg/mL solution in the same buffer, through an injection hole in the rim of the dish. The peptide was added to a final concentration of 2×10^{-7} M to ensure maximal pressure changes. The subphase was continuously stirred with a magnetic bar.

RESULTS

To test the effects of the colicin A pore-forming domain on the order of the acyl chain region of the phospholipids, the thermolytic peptide was added to dispersions of the total lipid extract of *E. coli* cells grown on [11,11-$^2H_2$]oleic acid, at both pH 4 and pH 6. ^{2}H NMR spectra were recorded and characterized by their quadrupolar splitting ($\Delta\nu_q$). The value of $\Delta\nu_q$ is related to the segmental order of the C–D bonds (Seelig, 1977) and can serve as a sensitive probe for acyl chain order in lipid bilayers. As shown in Figure 1A, a sample of phospholipids with no added protein at pH 4 gave rise to an axially symmetric ^{2}H NMR spectrum characterized by a $\Delta\nu_q$ of about 9.4 kHz. $\Delta\nu_q$ was found to be independent of the pH of the dispersion in the investigated region of pH 4–6 (not shown), indicating that lipid packing in the pure lipid system was not affected by pH changes in this range. The $\Delta\nu_q$ value of 9.4 kHz was also in good agreement with a previously reported value in a similar *E. coli* total lipid extract at pH 7.4 (Killian et al., 1992).

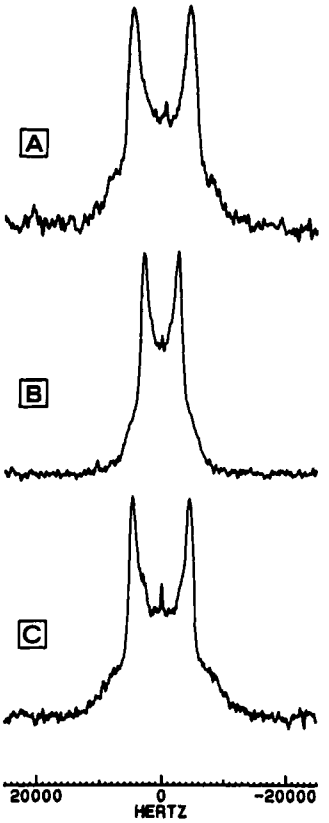

FIGURE 1: ^{2}H NMR spectra (46.0 MHz) of dispersions of the total lipid extract of *E. coli* K 1059 cells, grown on [11,11-$^2H_2$]oleic acid, in the absence of peptide at pH 4 (A) and with the thermolytic fragment of colicin A added at a 1/70 molar ratio of peptide to lipid at pH 4 (B) or pH 6 (C).

Addition of the thermolytic fragment of colicin A in a 1/70 molar ratio of peptide to lipid resulted in a large decrease of $\Delta\nu_q$ to 5.5 kHz when the peptide was added at pH 4 (Figure 1B). In contrast, no significant effect on $\Delta\nu_q$ of the lipid dispersion was observed when the peptide was added at pH 6.0 (Figure 1C). Upon lowering the pH in this sample to pH 4, again a large decrease of $\Delta\nu_q$ was obtained (not shown).

Control ^{31}P NMR measurements (e.g., see forthcoming Figure 5) demonstrated that, in all cases, the bilayer organisation was retained.

Next, the peptide to lipid molar ratio needed to observe the perturbing effects of the colicin A thermolytic fragment on lipid chain order at pH 4 was determined (Figure 2). At a low ratio of protein to lipid (below 1/150), the quadrupolar splitting gradually decreased with increasing peptide content. Upon further increasing the peptide/lipid ratios (1/100 and 1/70), the effect on $\Delta\nu_q$ was more pronounced. $\Delta\nu_q$ was reduced to 60% of its initial value at a 1/70 molar ratio. At pH 6, even at high peptide/lipid ratios, no significant effect of the peptide on $\Delta\nu_q$ was observed. At this pH only little binding was observed; 80% of the peptide remained in the supernatant after centrifugation of the hydrated phospholipid solution as indicated by the absorbance at 280 nm of the clarified supernatant (not shown). This is in agreement with previous observations that at pH 6 with 50 mM sodium chloride the thermolytic peptide binds very weakly to negatively charged phospholipids (Massotte et al., 1989; Frenette et al., 1989).

In order to determine the pK governing the effect observed on the deuterium spectra, the pH dependence of the effect of

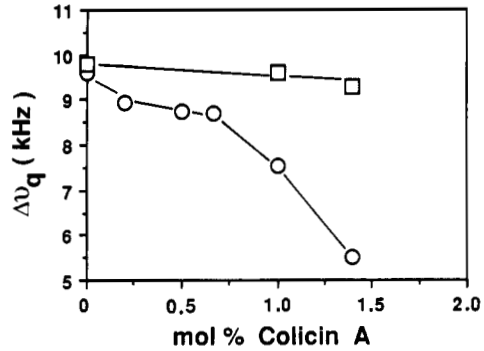

FIGURE 2: Effect of addition of the thermolytic fragment of colicin A on the quadrupolar splitting in dispersions of the total lipid extract of *E. coli* cells, grown on [11,11-$^2H_2$]oleic acid. Titrations were performed at pH 6 (□) and at pH 4 (○).

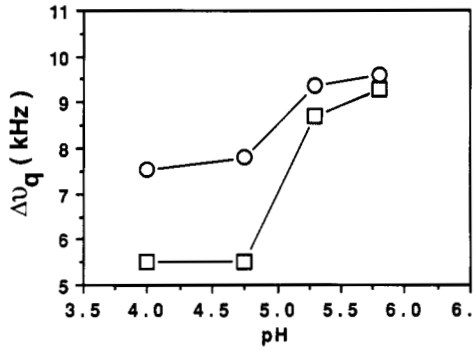

FIGURE 3: pH dependence of the effect of addition of the thermolytic fragment of colicin A on the quadrupolar splitting in dispersions of the total lipid extract of *E. coli* cells, grown on [11,11-$^2H_2$]oleic acid. The peptide was added in a molar ratio of peptide to lipid of 1/100 (○) and 1/70 (□).

the peptide on lipid order was further investigated using high peptide/lipid ratios of 1/100 and 1/70. As shown in Figure 3, the interaction of the peptide with lipid was highly dependent on the pH in a narrow region, between pH 4.75 and 5.3. The estimated p*K* is around 5. This value is in agreement with the pH-dependent appearance of a molten globular state of the thermolytic fragment, which has been reported to be a membrane insertion intermediate (van der Goot et al., 1991). However, the value for the p*K* governing the membrane insertion that we obtained and the one reported by van der Goot et al. (1991) are difficult to compare since different lipids, ionic strength, and buffer were used.

To investigate how this effect may be related to insertion of the peptide into the lipid phase, monolayer experiments were performed and the surface pressure increase of a monolayer of the same *E. coli* lipids upon peptide addition was investigated as a function of pH. Insertion of the peptide was found to occur in the same pH range as the peptide-induced decrease in $\Delta\nu_q$ reported above (Figure 4). The pressure increase was rapid and completed in 10–15 min. The maximal pressure increase obtained for the monolayers of *E. coli* total lipid extract was nearly 10 mN/m, leading to a final surface pressure of 40 mN/m at pH 4.5. This is similar to the maximal surface pressure demonstrated upon insertion of the thermolytic peptides in pure PG monolayers (Pattus et al., 1983). The pH range of the pressure change was between pH 5.1 and 4.75. The p*K* was found to be around 4.9, close to the value obtained with the thermolytic fragment and DOPG liposomes (van der Goot et al., 1991) and in accordance with the approximate p*K* value derived from the ^2H NMR data. At higher pH values, the thermolytic peptide did not insert into *E. coli* lipids at an initial pressure of 30 mN/m. The reversibility of the interaction was tested by injecting the peptide at pH 4.5 and increasing thereafter the pH to 6 (data not shown). We observed an immediate reduction by 65% of the peptide-induced pressure increase, demonstrating that the insertion is for a large part reversible.

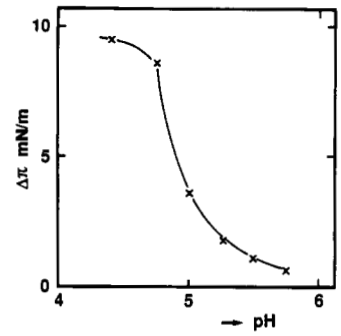

FIGURE 4: Thermolytic peptide–*E. coli* lipids interaction in monomolecular layers: pH dependence of surface pressure increase. The protein was injected in the subphase at 22 °C at an initial pressure of 30 mN/m.

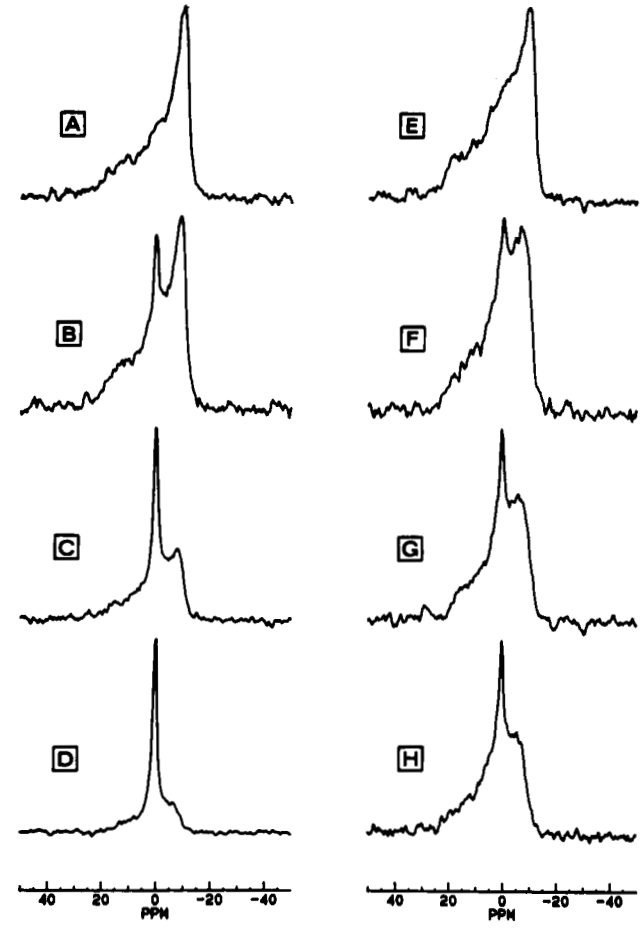

FIGURE 5: ^{31}P NMR spectra (121 MHz) of dispersions of the total lipid extract of *E. coli* K 1059 cells, grown on [11,11-$^2H_2$]oleic acid, in the absence of peptide (A–D) and with the thermolytic fragment of colicin A added at a 1/70 molar ratio of peptide to lipid (E–H) at 30 °C (A and E), 50 °C (B and F), 55 °C (C and G) and 60 °C (D and H). Equilibration times between successive temperatures were 10 min.

Next, we investigated the effects of insertion of the thermolytic peptide on membrane lipid organization. These experiments were performed at acidic pH, with a molar ratio of protein to lipids of 1/70, under which conditions the largest reduction of acyl chain order was observed. At 30 °C, the ^{31}P NMR spectrum of pure lipids (Figure 5A) is characterized by a low-field shoulder and a high-field peak, characteristic for lipids in a bilayer organization (Seelig, 1977; Cullis & De Kruijff, 1979). A similar spectrum was observed upon addition of the peptide (Figure 5E), demonstrating that the peptide

did not induce any major changes in lipid organization, although minor effects cannot be excluded. The chemical shift anisotropy observed in the ^{31}P NMR spectra is similar (~36 ppm) in the absence or presence of the peptide, indicating that the headgroups are not perturbed to a significant extent by interaction with the peptide (Figure 5A,E). These results also demonstrate that the large reduction in acyl chain order as observed by ^{2}H NMR is a direct result of the peptide/lipid interaction and cannot be due to the formation of smaller vesicles, which in principle also could result in a reduction of $\Delta\nu_q$ by an increased motional averaging as a result of lipid diffusion and/or vesicle tumbling (Burnell et al., 1980).

The effect of the thermolytic peptide on the phase behavior of the *E. coli* lipids was further investigated as function of temperature. It is well documented at pH 7.4 that pure *E. coli* lipids undergo a bilayer/isotropic phase transition at elevated temperatures (Killian et al., 1990, 1992). This ability to adopt nonbilayer structures is related to the "intrisic curvature" of the membrane lipids (Tate et al., 1991) and is believed to be important for membrane functioning (Lindblom & Rilfors, 1989; Killian et al., 1992). Figure 5A–D shows that, also at pH 4, a temperature-dependent change in lipid organization takes place. In the absence of the peptide, upon increasing the temperature to 50 °C and higher, gradually, an isotropic component is formed (Figure 5B–D). Similar behavior was observed at pH 6 (not shown) and was attributed to the formation of nonbilayer lipid structures with a type II surface curvature (Killian et al., 1990, 1992), which possibly represents a cubic phase (Lindblom & Rilfors, 1989). In the presence of the thermolytic fragment of colicin A, also a bilayer to nonbilayer transition occurred with increasing temperature. However, this transition was more gradual, as evidenced by the much smaller increase in intensity of the isotropic component upon raising the temperature as compared to the lipid sample in the absence of peptide (compare Figure 5 panels F–H and B–D). In the pure lipid sample it can be estimated that at 60 °C approximatively 75% of the lipids are undergoing fast isotropic motion, while this is only about 25% in the presence of the peptide.

These results demonstrate that insertion of the peptide in model membranes of the *E. coli* lipids stabilizes the bilayer organization.

DISCUSSION AND CONCLUSIONS

The ^{2}H NMR results show a pH-dependent pertubing effect of the thermolytic peptide of colicin A on the acyl chains in model membranes of the *E. coli* phospholipids. At acidic pH, the acyl chain order is strongly reduced while the lipid bilayer organization is maintained as was demonstrated by ^{31}P NMR measurements. The p*K* governing the effect on acyl chain order is correlated to the p*K* governing the insertion of the protein into monolayers of these lipids at physiological surface pressure of 30 mN/m. It is well documented that the interaction of the thermolytic peptide with membrane is dependent on the presence of negatively charged lipids (Pattus et al., 1983). The maximum surface pressure induced by the peptide in *E. coli* lipid monolayers and the pH dependence were similar to those observed upon insertion of the peptide in bilayers of the negatively charged phosphatidylglycerol (Pattus et al., 1983) indicating that the amount of 20% anionic lipids in *E. coli* extracts is sufficient to ensure maximum insertion of the protein.

The 40% decrease in quadrupolar splitting upon addition of the thermolytic fragment at low pH is most likely due to a pH-dependent membrane insertion of a part of the peptide. Such a strong effect on $\Delta\nu_q$ is highly unusual and so far has been observed for very few other peptides. One example is the mitochondrial precursor protein apocytochrome *c* upon interaction with bilayers, composed of dioleoylphosphatidylcholine and dioleoylphosphatidylserine, through which the peptide can at least in part spontaneously translocate (Jordi et al., 1990). Other examples are two synthetic positively charged pentapeptides, of which the acyl chain perturbing effect was attributed to a localization of the peptides at the membrane surface, intercalated between the lipid headgroups (De Kroon et al., 1991). Also, apocytochrome *c* has been shown to have a surface localization (Snel et al., 1991). In contrast, membrane-spanning peptides, such as gramicidin (Chupin et al., 1987; Watnick et al., 1990) or larger integral membrane proteins such as cytochrome oxidase (Tamm & Seelig, 1983) or rhodopsin (Bienvenue et al., 1982) have no large effect on acyl chain order as measured by ^{2}H NMR. Thus, the strong effect on $\Delta\nu_q$ of the thermolytic fragment of colicin A, as shown in the present study, suggests a surface localization of at least part of the peptide upon interaction with model membranes of *E. coli* phospholipids at low pH. However, our results do not indicate the precise location of the helices.

Such a localization could also explain the bilayer-stabilizing effect of the peptide. Nonbilayer structures, as adopted by the *E. coli* lipids at higher temperatures, are structures with a net concave (type II) surface curvature (Killian et al., 1992). A localization of the thermolytic fragment at the bilayer surface will inhibit the "curling" of the surface and thereby counteract the tendency to form nonbilayer lipid structures. This behavior was for instance demonstrated with the toxin melittin upon interaction with zwitterionic lipids (Batenburg et al., 1987). The effect of peptides on lipid organization is dependent on many factors, including the shape of the molecules, electrostatic interactions, and the membrane localization of the peptide (Batenburg & De Kruijff, 1988; Tournois & De Kruijff, 1991). The crucial role of peptide localization can be illustrated by the observation that the same toxin, melittin, upon interaction with lipids, in which it penetrates more deeply into the bilayer, has an opposite behavior and now promotes the formation of type II nonbilayer lipid structures (Batenburg et al., 1987). Similarly, hydrophobic peptides such as gramicidin and signal peptides, which partition into the hydrophobic interior of the bilayer, can induce the formation of a type II lipid organization (Killian et al., 1990).

Thus, both the ^{2}H and ^{31}P NMR data support a surface localization of the thermolytic fragment of colicin A upon interaction with *E. coli* lipids at low pH. According to the umbrella model of insertion of the peptide, a hydrophobic, α-helical hairpin (helices 8 and 9) inserts into the membrane, while the peripheral amphipathic helices have their axes parallel to the plane of the bilayer (Parker et al., 1989). Our results are compatible with this model, in which the surface-localized helices could then be responsible for the effects of the peptide on acyl chain order and for the bilayer stabilization by the thermolytic peptide.

However, recent fluorescence measurements indicated that, in contrast to what is proposed in the umbrella model, the hydrophobic hairpin of the thermolytic peptide does not insert as a perpendicular hairpin but remains packed to surface-localized helices (Lakey et al., 1992). Evidently, our ^{2}H and ^{31}P NMR data would be even better in agreement with this new model. Also, the monolayer experiments, which showed that the insertion of the thermolytic fragment is for a large part reversible, are in agreement with a surface localization of all helices.

It is interesting to compare this behavior with the effect of diphtheria toxin on surface pressure in lipid monolayers, which is not reversed by returning the pH to neutrality (Demel et al., 1991). The transmembrane fragment of diphtheria toxin has a rather similar structure as the thermolytic fragment of colicin A (Chloe et al., 1992), and the proposed mode of insertion is comparable to the umbrella model, described for colicin A, except that it involves two pairs of hydrophobic helices. It is possible that a more extensive hydrophobic interaction in the case of diphteria toxin is responsible for the difference in the interaction of these peptides with lipid monolayers and leads to insertion of both pairs of helices in the case of diphteria toxin, while for the colicin A thermolytic fragment all helices are surface localized. Most likely, it is this surface localization of the helices that is responsible for the large perturbing effect of the colicin A thermolytic fragment on lipid chain packing, as observed in this study. We propose that this perturbation of chain packing plays a role in facilitating the insertion and organization into the membrane of the structure required for the pore formation of colicin A in the next voltage-dependent step.

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