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# Solid-state NMR studies of the membrane-bound closed state of the colicin E1 channel domain in lipid bilayers

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**Abstract**

The colicin E1 channel polypeptide was shown to be organized anisotropically in membranes by solid-state NMR analysis of samples of uniformly $^{15}$N-labeled protein in oriented planar phospholipid bilayers. The 190 residue C-terminal colicin E1 channel domain is the largest polypeptide to have been characterized by $^{15}$N solid-state NMR spectroscopy in oriented membrane bilayers. The $^{15}$N-NMR spectra of the colicin E1 show that: (1) the structure and dynamics are independent of anionic lipid content in both oriented and unoriented samples; (2) assuming the secondary structure of the polypeptide is helical, there are both *trans*-membrane and in-plane helical segments; (3) *trans*-membrane helices account for approximately 20–25% of the channel polypeptide, which is equivalent to 38–48 residues of the 190-residue polypeptide. The results of the two-dimensional PISEMA spectrum are interpreted in terms of a single *trans*-membrane helical hairpin inserted into the bilayer from each channel molecule. These data are also consistent with this helical hairpin being derived from the 38-residue hydrophobic segment near the C-terminus of the colicin E1 channel polypeptide.

**Keywords:** colicin, ion channel; membrane proteins; membranes; protein import; solid-state NMR; voltage-gated channel

Colicin E1 is a 522-residue bactericidal protein. It exerts its lethal effect on sensitive *E. coli* cells by forming an ion channel that is sufficiently conductive to depolarize and deenergize the cytoplasmic membrane (Gould & Cramer, 1977; Bullock et al., 1983). Colicin E1 is a member of a family of at least seven channel-forming colicins (Braun & Pilsl, 1995; Cramer et al., 1995), that utilize metabolite- and phage-binding receptors in the outer membrane to enter the cell, and at least two different intra- and inter-membrane protein systems, *tol* and *ton*, to translocate from the outer to the inner membrane (Cramer et al., 1995). Studies of colicins A, E1, Ia, and N from this family have been particularly useful in providing information on structure–function relationships, as have studies on toxin molecules (Parker & Pattus, 1993). The electrical properties of the entire colicin molecule, as well as of its channel domain alone, can be characterized in vitro using planar bilayer or patch-clamp techniques. Channel activity similar to that of the intact 522-residue colicin molecule is found in a 190-residue C-terminal domain (Dankert et al., 1982; Bullock et al., 1983). The structure of the colicin channel domains of colicins A (Parker et al., 1989, 1992) and E1 (Elkins et al., 1997), as well as the entire colicin Ia molecule (Wiener et al., 1997), have been determined by X-ray crystallography. The colicin channel domain undergoes the transition from a soluble to a membrane-bound ion-conductive open-channel state through an intermediate membrane-bound state that is closed to ion flow in the absence of a membrane potential. The binding of the channel polypeptide to liposomes in this intermediate state is characterized by a high-affinity, a $K_d \sim 10$ nM, under conditions of optimum activity (Heymann et al., 1996; Zakharov et al., 1996). To understand the pathway and mechanisms by which these colicins undergo large conformational changes that accompany the transitions from (1) the soluble to the membrane-bound state; and (2) the closed- to the open-channel state, it is essential to characterize the structural properties of the bound intermediate state.

The existence of a hydrophobic 38–48 residue segment near the C-terminus of the channel polypeptide in all channel forming colicins (Table 1) led to the proposal that the channel domain is partly

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*Abbreviations:* DLPC, DMPC, dilauryl-, dimyristoyl-phosphatidylcholine; PG, phosphatidylglycerol; DMPG, dimyristoylphosphatidylglycerol; DOPG, dioleolylphosphatidylglycerol; NMR, nuclear magnetic resonance; OD, optical density; P190, C-terminal 190 residue colicin E1 polypeptide; PISEMA, polarization inversion spin exchange at the magic angle.

**Table 1.** *Sequence of the C-terminal domain of the colicin E1 ion channel showing the 10 helical segments in the atomic structure (Elkins et al., 1997)*[a]

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        340         350         360         370         380
          |           |           |           |           |
                 **         *       *  *        * * *     *
ENLKKAQNNL LNSQIKDAVD ATVSFYQTLT EKYGEKYSKM AQELADKSKG
                   [-----Helix 1----]  [---Helix 2---]

        390         400         410         420         430
          |           |           |           |           |
      * ***     ** *      **     **    *  **
KKIGNVNEAL AAFEKYKDVL NKKFSKADRD AIFNALASVK YDDWAKHLDQ
     [----Helix 3---]       [--Helix 4-]      [Helix] [Helix
                                                5a      5b

        440         450         460         470         480
          |           |           |           |           |
  *     *         *       *   *  * ** *   *
FAKYLKITGH VSFGYDVVSD ILKIKDTGDW KPLFLTLEKK AADAGVSYVV
---]              [--Helix 6-]      [--Helix 7-]    [----

        490         500         510         520
          |           |           |           |
   **    *    *  *                       *    **
ALLFSLLAGT TLGIWGIAIV TGILCSYIDK NKLNTINEVL GI 522
-Helix 8]       [---Helix 9--]          [Helix]
                                          10
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[a]The hydrophobic helices, 8 and 9, are underlined.
*Residues conserved in all E and A type colicins.

anchored to the membrane by a *trans*-membrane hydrophobic helical hairpin that is inserted spontaneously upon binding of the channel polypeptide to the membrane. The insertion of the remainder of the *trans*-membrane charged/amphiphilic helices would then be driven by a *trans*-membrane potential (Cleveland et al., 1983; Merrill & Cramer, 1990; Slatin et al., 1994). The localization of the hydrophobic hairpin of the open-channel form of the polypeptide within the membrane bilayer of the *E. coli* cytoplasmic membrane has been inferred from the cytotoxicity pattern of a large number of site-directed charge substitution mutants (Song et al., 1991), analysis of the environments of spin-labeled residues of the colicin E1 channel polypeptide inserted into DLPC membranes (Shin et al., 1993), and streptavidin trapping on the *trans*-side of the membrane of colicin $I_a$, biotin-labeled at a Cys residue located at the putative apex of the hydrophobic hairpin (Kienker et al., 1997). Although a number of studies on the state of the 204-residue colicin A channel polypeptide bound to liposomes also led to the conclusion that the channel was anchored in the membrane by the C-terminal hydrophobic domain (Fig. 1B) (the "umbrella" model; e.g., Parker et al., 1992), subsequent studies on colicin A indicated that this hydrophobic hairpin may not be inserted spontaneously, and may be bound at the membrane surface (Fig. 1A, "penknife" model) (e.g., Lakey et al., 1993; Duché et al., 1994).

Solid-state NMR studies of oriented samples have been successfully used to distinguish between in-plane and *trans*-membrane orientations of a variety of 20–25-residue helical peptides associated with phospholipid bilayers (Bechinger et al., 1996). Solid-state NMR spectroscopy can also be used to identify the orientations of helical segments of larger membrane proteins (McDonnell et al., 1993; Cross & Opella, 1994), and this could be used to answer some of the structural and mechanistic questions regarding

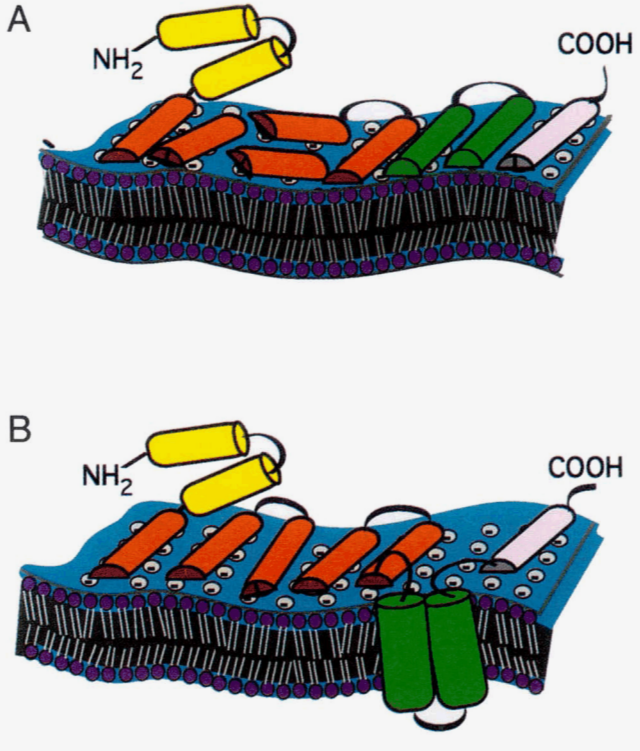


**Fig. 1.** Schematic representation of (**A**) "penknife" and (**B**) "umbrella" models for surface-bound closed state (i.e., no membrane potential) of colicin E1 channel polypeptide. Surface-bound helices are embedded in the membrane interfacial layer (Krishtalik et al., 1993; White & Wimley, 1994), and are shown slightly protruding to illustrate their surface location.

the colicin channel mentioned above. This NMR approach to protein structure determination relies on the established relationships between the frequencies associated with spin interactions at the amide sites (e.g., $^{15}N$ chemical shift) of peptide bonds and the orientations of those bonds relative to the direction of the applied magnetic field of the NMR spectrometer (Opella et al., 1987). If the residue is known to be part of an $\alpha$-helix, chemical shift measurements can be directly interpreted in terms of the orientations of the helices with respect to the plane of the membrane bilayer (Bechinger et al., 1991).

Amphipathic helical peptides have been found to have either orthogonal or in-plane orientations in membrane bilayers, and a model hydrophobic peptide was found to be *trans*-membrane, as expected (Bechinger et al., 1996). The membrane-bound forms of the coat proteins of two different filamentous bacteriophages, Pf1 (46 residues) (Shon et al., 1991) and fd (50 residues) (McDonnell et al., 1993) have been characterized by solid-state NMR spectroscopy. With the advent of multidimensional solid-state NMR experiments it is possible to resolve individual resonances using uniformly $^{15}N$-labeled samples (Marassi et al., 1997). Both proteins have a long hydrophobic helix, which was found to be *trans*-membrane, and a short amphipathic helix, which was found to be oriented parallel to the plane of the membrane bilayers. Simply establishing that a helical peptide has a *trans*-membrane or in-plane orientation provides significant information about its structural and functional roles. The sorting of peptides or segments of proteins into two classes, surface bound with in-plane alignments, and those with *trans*-membrane orientations, is consistent with what is known about the architectural principles of helical membrane proteins.

Solid-state NMR spectroscopy on oriented samples is capable of determining the three-dimensional structures of proteins (Opella et al., 1987), including membrane proteins in lipid bilayers (Ramamoorthy et al., 1995; Marassi et al., 1997). In the full application of the method, the measurement of two or three spectral parameters for each peptide group is needed to determine its orientation without additional information from other sources. This is different from the results described here, where only a single measurement is obtained and additional information, namely that the labeled site is in the helix, is utilized in the qualitative structural interpretations (Bechinger et al., 1991, 1996).

Following the protocol developed using smaller peptides and proteins, solid-state $^{15}N$-NMR experiments were carried out on samples of uniformly $^{15}N$-labeled 190-residue C-terminal colicin E1 polypeptide bound to phospholipid bilayers. Spectra were obtained from both oriented and unoriented samples. Among the most important conclusions of these experimental studies is that 20–25% of the residues of this polypeptide exist in *trans*-membrane helices, which is consistent with the "umbrella" model for binding and insertion of the colicin channel polypeptide to membrane bilayers.

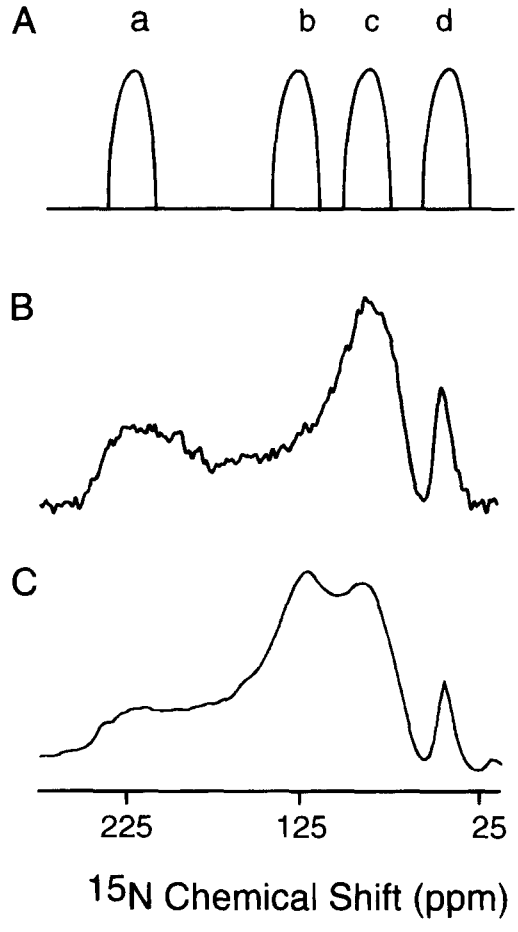


**Fig. 2.** Solid-state $^{15}N$-NMR spectra of protein sites. **A:** Simulated resonance lines based on the known $^{15}N$ chemical shift properties. a) Resonance frequency associated with an N–H bond parallel to the direction of the applied magnetic field. b) Resonance frequency associated with an isotropically averaged (mobile) amide group. c) Resonance frequency associated with an N–H bond perpendicular to the direction of the applied magnetic field. d) Resonance frequency associated with amino groups. **B:** Experimental spectrum obtained on an oriented sample of uniformly $^{15}N$-labeled colicin E1 (P190) in fully hydrated phospholipid bilayers. The sample consisted of 6 mg of protein in a phospholipid mixture consisting of 80% DMPC (96 mg) and 20% DMPG (24 mg); temperature, 28 °C. The sample was oriented on a stack of twenty-five 11 × 11 × 0.07 mm glass plates and the spectrum was obtained using a flat-coil probe. Transients (13,400) were co-added prior to Fourier transformation to obtain the spectrum. **C:** Experimental spectrum obtained on an unoriented sample of uniformly $^{15}N$-labeled colicin E1 (p190) in fully hydrated phospholipid bilayers. The sample consisted of 5 mg of protein in a phospholipid mixture consisting of 80% DMPC (96 mg) and 20% DMPG (24 mg) in a sealed glass sample tube in a solenoidal coil probe. Transients (20,000) were co-added prior to Fourier transformation; temperature, 55 °C.

## Results

The experimental solid-state NMR spectra shown in the figures were obtained from oriented and unoriented samples of uniformly $^{15}N$-labeled colicin E1 channel P190 polypeptide in fully hydrated phospholipid bilayers consisting of 80% DMPC and 20% DMPG. The amount of acidic lipid is approximately physiological for the *E. coli* cytoplasmic membrane (Shibuya, 1992). The spectra from oriented samples (Fig. 2B) are indistinguishable from those obtained with phospholipid bilayers of either 100% DMPC or 100% DMPG. Samples prepared by incorporation of colicn E1 into sonicated vesicles in an aqueous buffer showed a similar oriented spectrum. This indicates that the protein behaves the same when extracted from $CHCl_3$ into lipids and then hydrated or directly incorporated into preformed vesicles in aqueous solution. The spectrum of an unoriented sample in Figure 2C was obtained at the relatively high temperature of 55°, well above any domain phase transition temperature of the lipid mixture.

Unoriented samples provide valuable information about protein dynamics because there are pronounced effects of motional averaging on powder pattern spectra (Opella, 1985). The spectrum from the unoriented sample of colicin E1 polypeptide in Figure 2C shows three distinct kinds of signal intensity. The chemical shifts are referenced to external liquid ammonia at 0 ppm. At the upfield end, near 38 ppm, is a relatively narrow peak from the various amino groups in the protein, also present in the oriented spectrum in Figure 2B, and labeled "d" in the simulated "stick" spectrum in Figure 2A. This peak would be narrow, regardless of the dynamics of the amino groups because of the small breadth of the chemical shift anisotropy powder pattern for amino nitrogens, and it does not contribute to our analysis of this protein. In contrast, amide nitrogens have much broader powder patterns with approximately 170 ppm separating the downfield edge (near 235 ppm) and the upfield edge (near 60 ppm). This can be seen in the breadth of the experimental spectrum of the unoriented sample in Figure 2C. The characteristic powder pattern shape is present, with no evidence of narrowing due to motional averaging, in spite of the 55 °C temperature. However, the spectrum has a third type of resonance intensity, which is relatively narrow and centered at 117 ppm because of extensive motional averaging, superimposed on the broad powder pattern. 117 ppm is approximately the isotropic resonance position, the average of the three principal elements of the chemical shift tensor for the amide nitrogen. In this context the isotropic intensity is due to a significant number of the backbone amide sites in the polypeptide undergoing large amplitude motions more frequently than the 10 kHz breadth of the amide $^{15}N$ chemical shift powder pattern.

There are obvious qualitative differences between the spectrum from an unoriented (Fig. 2C) and an oriented sample (Fig. 2B). Most significantly, in the oriented sample there is a substantial increase in the resonance intensity near the downfield edge of the spectrum (near 235 ppm), which is characteristic of peptides and proteins with *trans*-membrane helices (Bechinger et al., 1991, 1996). This demonstrates that the polypeptide is oriented along with the phospholipids in the samples prepared between glass plates. There are four major peaks (labeled a–d), in the simulated "stick" spectrum in Figure 2A that correspond to the main resonance bands observed in the experimental spectrum from the oriented sample. These peaks correspond to (d) amino groups; (b) mobile amide groups; (a) rigid, structured amide groups with their N–H bonds approximately aligned with the direction of the applied magnetic field; and (c) amide groups with their N–H bonds aligned perpendicular to the direction of the field. The data demonstrate that the protein definitely has *trans*-membrane helices (peak a), in-plane helices (peak c), and mobile loops, and/or terminal regions (peak b), as is typical of helical membrane proteins. A $^{31}P$-NMR spectrum of the colicin E1 sample oriented between glass plates gave the expected result for bilayers. The majority of intensity is at 26 ppm with a minor component at −13 ppm. The axially symmetric powder pattern for an unoriented phospholipid bilayer would give the perpendicular component at 26 ppm with a parallel component at −13 ppm relative to external concentrated phosphoric acid at 0 ppm. This indicates the bilayer is oriented with the bilayer parallel to the field. The signal at −13 ppm increases over the lifetime of the sample (weeks), indicative of some loss of orientation presumably due to rf heating or other factors.

The two-dimensional PISEMA spectrum of colicin E1 oriented on glass plates in 80% DMPC and 20% DMPG is shown in Figure 3. This experiment correlates $^{15}N$ chemical shift with $^{15}N$-$^{1}H$ dipolar coupling frequencies (Wu et al., 1994), both of which vary with the molecular orientation in characteristic ways. At the chemical shift values near 235 ppm the dipolar splittings are near maximal, ~10 kHz. This splitting is indicative of an N–H bond vector aligned parallel to the magnetic field. Remarkably, there are many resolved individual resonances in this region of the two-dimensional spectrum that can only arise from residues in *trans*-membrane helices. At the isotropic chemical shift, 117 ppm, near zero dipolar splittings indicate the dipolar interaction is averaged by motion. Additional intensity at zero frequency dipolar splitting and upfield in chemical shift from the isotropic position can occur as an artifact in the PISEMA spectrum due to the loss of the homonuclear decoupling of the protons at these $^{15}N$ frequencies. The majority of intensity is centered at 80 ppm, where the dipolar splitting is one-half the maximal value (~5 kHz). This indicates that the N–H bond vectors are oriented perpendicular to the applied magnetic field. From the distribution of resonances in this spectrum it can be concluded that ~20% of the residues are in helices oriented *trans*-membrane, a small percentage are motionally averaged, and the remaining ~70% are in helices in the plane of the bilayers. This is in agreement with the findings based on the one-dimensional solid-state NMR spectra in Figure 2.

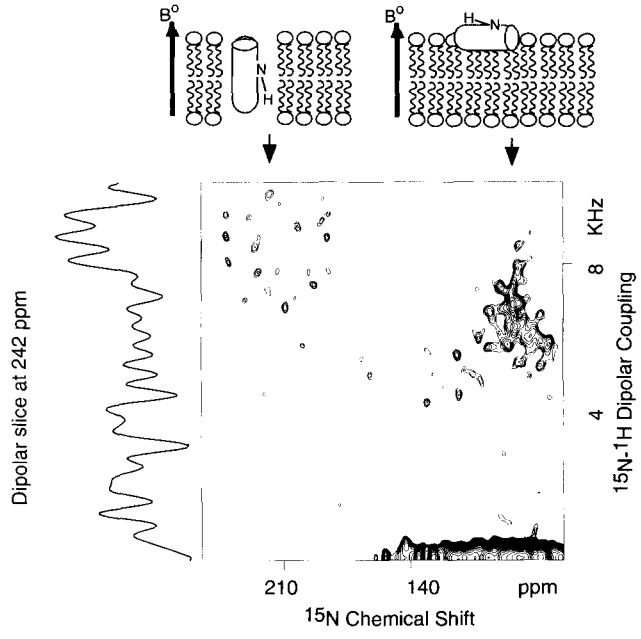


**Fig. 3.** Solid-state two-dimensional $^{15}N$ chemical shift/$^{15}N$-$^{1}H$ dipolar coupling PISEMA spectrum of an oriented sample of uniformly $^{15}N$-labeled colicin E1 P190 in fully hydrated phospholipid bilayers. The sample contained 6 mg of protein in a 80% DMPC and 20% DMPG mixture, temperature, 28 °C. The sample was oriented on a stack of twenty-five 11 × 11 × 0.07 mm glass plates in a flat coil probe double tuned to 700.39 MHz and 70.97 MHz. The PISEMA spectrum was obtained using a cross-polarized field strength of 52 kHz. The $^{1}H$ decoupling field was 62.5 kHz. Transients (256) were co-added for each of 64 $T_1$ points. The data was zero filled to form a 1,024 × 512 matrix using a Gaussian multiplication of 300 Hz in $T_2$ and a Gaussian multiplication of 300 Hz in $T_1$. A dipolar slice taken at 242 ppm is indicated at the left of the two-dimensional spectrum. The region of the spectrum around 230 ppm represents intensity from amides in *trans*-membrane helix, while the intensity around 80 ppm represents amides in a helix in the plane of the bilayers as indicated by the diagram.

## Discussion

In previous studies, the orientations of helical segments of polypeptides with 20–50 residues were determined with solid-state NMR experiments on $^{15}N$-labeled samples in oriented lipid bilayers. The 190-residue C-terminal colicin E1 channel domain is the largest polypeptide that has been shown by $^{15}N$ solid-state NMR spectroscopy to be anisotropically oriented in membrane bilayers. Although individual or overlapped resonances have not yet been assigned to specific residues in the polypeptides, several major conclusions can be derived from their properties. (1) The structure and dynamics of the colicin channel domain are independent of lipid composition, because very similar spectra were obtained with the polypeptide in neutral DMPC, acidic DMPG, and DMPC/DMPG bilayers. (2) The colicin channel polypeptide consists mainly of rigid structured sections, although it has some mobile residues, most likely in loops between helices and the N-terminal region that is quite accessible to proteases (Zhang & Cramer, 1992). This conclusion is based on the observation of isotropic resonance intensity superimposed on the powder pattern in the spectrum in Figure 2C as well as in the oriented spectrum in Figure 2B. (3) Assuming the secondary structure of the polypeptide is primarily helical, there are both *trans*-membrane and in-plane helical segments in the polypeptide. This is based not only on the presence of peaks "a" and "d" in the one-dimensional oriented spectrum, but also on resonances resonances from three of the residues that are resolved in the dipolar slice shown in Figure 3. (4) Approximately 20–25% percent of the polypeptide, equivalent to 38–48 residues of the 190-residue channel polypeptide, must be allocated to the *trans*-membrane helical component to account for the relative intensities observed in the solid-state NMR spectra. This finding is consistent with each channel molecule having a single pair of *trans*-membrane helices inserted into the bilayer. It is also consistent with this helical hairpin being derived from the 38-residue hydrophobic segment near the C-terminus of the colicin E1 channel polypeptide.

In addition to these NMR data, *trans*-bilayer membrane insertion of the hydrophobic hairpin in vitro in the absence of a membrane potential is implied by (1) the absence of increased insertion of this channel domain into the bilayer in the presence of a membrane potential, measured by labeling with impermeant and photoaffinity lipophilic probes (Merrill & Cramer, 1990); (2) determination of an appreciable content of *trans*-bilayer $\alpha$-helix, consistent with at least one *trans*-bilayer helical hairpin, was implied for both colicins E1 (Rath et al., 1991) and A (Goormaghtigh et al., 1991) in oriented membranes by dichroism of the helix amide I transition monitored by infrared spectroscopy; (3) the recent finding that the secondary structure content of membrane bound colicin E1 P190 is approximately 90% $\alpha$-helical (Zakharov et al., 1997); (4) *trans*-side labeling of the hydrophobic hairpin of a fraction of the colicin $I_a$ molecules inserted into the bilayer (Kienker et al., 1997).

The model for a surface-bound state including a spontaneously inserted hydrophobic hairpin is structurally consistent with the X-ray crystal structures of the colicin A (Parker et al., 1989, 1992) and E1 (Elkins et al., 1997) channel polypeptides that showed the putative *trans*-membrane hydrophobic hairpin, VIII–IX, as its hydrophobic core helices. The X-ray crystal structure of the colicin A channel polypeptide originally led to the articulation of the "umbrella" model for the surface-bound state of the colicin. In this model, the preformed and membrane-inserted *trans*-membrane hydrophobic hairpin is the "umbrella" handle, and the "umbrella covering" bound at the membrane surface consists of the other helices (Fig. 1) preformed in the soluble channel polypeptide (Parker & Pattus, 1993). The umbrella model for colicin A was consistent with: (1) the above-mentioned studies on colicin E1, (2) studies on the interaction of the colicin A and its channel domain with lipids and membranes, which demonstrated a large molecular area (2,000 Å/molecule) for the colicin inserted into PG monolayers (Pattus et al., 1983), and a preference of 12–14 carbons for lipid fatty acid chain length, in PG lipids with which it forms disc-like structures (Massotte et al., 1989); (3) the time course of quenching of channel Trp fluorescence by brominated lipid in DOPG membranes (Gonzalez-Mañas et al., 1992).

However, additional studies on the *in vitro* surface topology of the colicin A channel polypeptide led to an alternate model for the membrane-bound state of the channel polypeptide, the "penknife" model, in which the hydrophobic hairpin is not spontaneously inserted into the bilayer in the absence of a membrane potential, but remains bound at, or near, the membrane surface, with the helix axes approximately parallel to the plane of the membrane and to the other uninserted helices. This model is based on (1) the absence of large changes in fluorescence energy transfer from the three Trp residues to three different labeled Cys residues upon binding colicin to discoidal-like lipid structures containing 100% negatively charged PG lipid (Lakey et al., 1993); (2) a large effect on acyl chain order measured by $^2H$-NMR caused by channel peptide binding to membranes derived from labeled *E. coli*, indicative of interaction with lipid head groups (Geli et al., 1992); (3) proteolytic cleavage of the hydrophobic hairpin region of the colicin A channel peptide bound to 100% DMPG membranes (Massotte et al., 1993), but at a higher lipid:protein ratio (100:1) than was used to probe accessibility of this region in colicins E1 and Ia, described above; (4) the neutron scattering profile of channel polypeptide bound to 100% DMPG membranes at a lipid:protein ratio of 25:1 (Jeunteur & Pattus, 1994); and (5) the effect of interhelix disulfide bridges on insertion (or binding) of colicin channel bound to brominated DOPG lipid structures (Duché et al., 1994). These experiments can individually be critically analyzed: (1) in the fluorescence energy transfer experiment, one does not know the distribution of donor–acceptor distances in the initial surface-bound state; (2) the $^2H$-NMR studies do not exclude insertion of the hydrophobic hairpin into the bilayer, which would have only a weak effect on acyl chain order; (3) the protein:lipid ratio in both the proteolysis and neutron scattering experiments were very high; (4) these experiments also have the common feature that they utilize 100% anionic lipid. However, the total weight of the data suggests an absence of spontaneous insertion of the hydrophobic hairpin in a *trans*-membrane conformation under many experimental circumstances.

The analysis of the solid-state NMR spectra obtained on oriented samples of uniformly $^{15}N$-labeled colicin channel polypeptide in hydrated lipid bilayers indicate that approximately 20–25% of the polypeptide, equivalent to 38–48 residues, inserts into phospholipid bilayers with the structural properties of *trans*-membrane helices. These structural data support a model ("umbrella," Fig. 1B) for binding and insertion of the colicin channel-forming polypeptides in which the hydrophobic hairpin can spontaneously insert into the membrane bilayer and thereby serve as an anchor for the insertion of the colicin E1 channel polypeptide in phospholipid bilayers. Considering these data and those of Kienker et al. (1997) on the one hand, and on the other those cited above that imply a "penknife" model, the penknife model (Fig. 1A) may be an intermediate in the formation of the inserted "umbrella" state (Fig. 1B). It has been pos-

sible to observe the latter state in vitro under the conditions of the solid-state NMR experiment.

## Materials and methods

### *Production of $^{15}$N-labeled colicin E1 channel peptide (P190)*

A prototrophic strain, BM13711 (B. Wanner, Purdue University) harboring the plasmid pSKHY was used to produce $^{15}$N-labeled P190. Direct expression of the C-terminal channel-forming domain of colicin E1 by pSKHY avoids possible contamination by residual protease if the latter is used to form channel polypeptide by cleavage of the colicin E1 molecule. The 190-residue peptide produced by pSKHY contains three more residues than the tryptic fragment, which was originally used to define the colicin E1 channel domain. The first five residues of the P190 polypeptide are M-E-T-A-E, whereas the corresponding residues in the colicin E1 molecule are L-K-K-A-Q (Elkins et al., 1994).

Ten milliliters of an overnight culture of BM13711(pSKHY) were used to inoculate 1 L of minimal media containing (g/L): $K_2HPO_4$, 7.0; $NaH_2PO_4$, 3.0; NaCl, 0.5; $^{15}NH_4Cl$ (Cambridge Isotope Laboratory), 1.0; glucose, 2.0; 8.3 mg $MgSO_4$, 100 mg ampicillin and trace metals (Anraku, 1967). The overnight cultures were grown in YT media [8 g Tryptone (Difco), 5 g Yeast Extract (Difco), 2.5 g NaCl]. According to batch data from Difco, the Tryptone and Yeast Extract contain 14 and 12% nitrogen (w/w), respectively. Thus, with a 1:100 dilution of the overnight culture, the fractional content of $^{15}$N is >99%. The cells were grown at 37° with aeration by shaking to an $OD_{650}$ of approximately 1.0 and then induced with 0.5 mg of mitomycin C for 4 h. The cells were then harvested and resuspended in 50 mM sodium acetate, pH 6.0, and broken in a French pressure cell. The resulting lysate was centrifuged at 14,500 × *g* (20 min), and the supernatant applied to a CM-50 Sephadex (Pharmacia) column equilibrated with 50 mM sodium acetate, pH 6.0. The column was washed overnight with sodium acetate buffer, pH 6.0, and the P190 was eluted from the column with 50 mM sodium acetate, pH 6.0, and 0.1 M NaCl. The fractions containing the P190 were pooled, concentrated and dialyzed into $H_2O$ using a Pro-Di-Con apparatus (Spectrum). Concentrated P190 was then lyophilized to facilitate resuspension in organic solvent.

### *Solid-state NMR spectra*

Spectra were obtained on oriented and unoriented samples of uniformly $^{15}$N-labeled C-terminal colicin E1 channel polypeptide (P190) in fully hydrated phospholipid bilayers. The spectra were obtained on home-built four-channel spectrometers with a wide-bore Magnex 550/89 magnet or a Magnex 750/62 magnet. The probes were single coil-double tuned for $^1$H resonance frequency of 550 or 700 MHz and $^{15}$N resonance frequency of 55.7 or 70.9 MHz. The oriented samples consisted of 25 stacked glass plates with the protein containing bilayers sandwiched between them, inserted in the square-coil of the probe. The unoriented samples were in a sealed holder and placed in a solenoidal coil probe. The colicin peptide was solubilized into DMPC and DMPG lipids in $CHCl_3$ with 1,1,1,3,3,3-hexafluoro-2-propanol added dropwise until the solution cleared with bath sonication. The solution was then placed on twenty-five 11 × 11 × 0.07 mm plates allowed to dry and then stacked and hydrated at 94% relative humidity at 42 °C. The spectra were obtained by spin-lock cross-polarization with phase alternation of three intervals of 333 $\mu$s giving a total single contact mix time of 1 ms. The recycle delay was 5 s and the $^1$H 90° pulse length was typically 4.8 $\mu$s. In the two-dimensional PISEMA spectrum a 1-ms cross-polarization mix was followed by 64 increments of a flip-flop Lee-Goldberg homonuclear $^1$H irradiation during the evolution period (Wu et al., 1994).

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## References

Anraku Y. 1967. The reduction and restoration of galactose transport in osmotically shocked cells of *Escherichia coli*. *J Biol Chem 242*:793–800.

Bechinger B, Gierasch LM, Montal M, Zasloff M, Opella SJ. 1996. Orientations of helical peptides in membrane bilayers by solid-state NMR spectroscopy. *Solid-state NMR 7*:185–191.

Bechinger B, Kim Y, Chirlian LE, Gesell J, Neumann JM, Montal M, Tomich J, Zasloff M, Opella SJ. 1991 Orientations of amphipathic helical peptides in membrane bilayers determined by solid–state NMR spectroscopy. *J Biomolec NMR 1*:167–173.

Braun V, Pilsl H. 1995. Novel colicin 10: Assignment of four domains to TonB and TolC-dependent uptake via the Tsx receptor and pore formation. *Mol Microbiol 16*:57–67.

Bullock JO, Cohen FS, Dankert JR, Cramer WA. 1983. Comparison of the macroscopic and single channel conductance properties of colicin E1 and its COOH-terminal tryptic peptide. *J Biol Chem 258*:9908–9912.

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

Cramer WA, Heymann JB, Schendel SL, Deriy BN, Cohen FS, Elkins PA, Stauffacher CV. 1995. Structure–function of the channel-forming colicins. *Annu Rev Biophys Biomol Struct 24*:611–641.

Cross TA, Opella SJ. 1994. Solid-state NMR structural studies of peptides and proteins in membranes. *Curr Opin Struct Biol 4*:572–581.

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

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

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

Elkins P, Bunker A, Cramer WA, Stauffacher CV. 1997. The crystal structure of the channel-forming domain of colicin E1. *Structure 5*:443–458.

Geli V, Koorengevel MC, Demel RA. 1992. Acidic interaction of the colicin A pore-forming domain with model membranes of *Escherichia coli* lipids results in a large perturbation of acyl chain order and stabilization of the bilayer. *Biochemistry 31*:11089–11094.

Gonzalez-Mañas JM, Lakey JH, Pattus F. 1992. Brominated phospholipids as a tool for monitoring the membrane insertion of colicin A. *Biochemistry 31*:7294–7300.

Goormaghtigh E, Vigneron L, Knibiehler M, Lazdunski C, Ruysschaert JM. 1991. Secondary structure of the membrane-bound form of the pore-forming domain of colicin A. *Eur J Biochem 202*:1299–1305.

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

Heymann JB, Zakharov SD, Zhang Y-L, Cramer WA. 1996. Characterization of electrostatic and non-electrostatic protein-membrane binding interactions. *Biochemistry 35*:2717–2725.

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

Kienker P, Qiu X-Q, Slatin SR, Finkelstein A, Jakes K. 1997. Membrane insertion of the colicin $I_a$ hydrophobic hairpin. *J Memb Biol 157*:27–37.

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

Marassi FM, Ramamoorthy R, Opella SJ. 1997. Complete resolution of the solid-state NMR spectrum of a uniformly $^{15}$N-labeled membrane protein in phospholipid bilayers. *Proc Natl Acad Sci USA 94*:8551–8556.

Massotte D, Dasseux J-L, Sauve P, Cyrklaff M, Leonard K, Pattus F. 1989. Interaction of the pore-forming domain of colicin A with phospholipid vesicles. *Biochemistry 28*:7713–7719.

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

McDonnell PA, Shon K, Kim Y, Opella SJ. 1993. fd coat protein structure in membrane environments. *J Mol Biol 233*:447–463.

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

Opella SJ, Stewart PL, Valentine KG. 1987. Protein structure by solid-state NMR spectroscopy. *Q Rev Biophys 19*:7–49.

Opella SJ. 1985. Protein dynamics by solid-state NMR. *Methods Enzymol 131*:327–361.

Parker MW, Pattus F. 1993. Rendering a membrane protein soluble in water: A common packing motif in bacterial protein toxins. *Trends Biosci 18*:391–395.

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

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

Pattus F, Martinez MC, Dargen B, Carvard D, Verger R, Lazdunski C. 1983. Interaction of colicin A with phospholipid monolayers and liposomes. *Biochemistry 22*:5698–5703.

Ramamoorthy A, Marassi FM, Zasloff M, Opella SJ. 1995. Three-dimensional solid-state NMR spectroscopy of a peptide oriented in membrane bilayers. *J Biomol NMR 6*:329–334.

Rath P, Bousché O, Merrill AR, Cramer WA, Rothschild KJ. 1991. Fourier transform infrared evidence for a predominantly $\alpha$–helical structure of the membrane bound channel forming COOH-terminal peptide of colicin E1. *Biophys J 59*:516–522.

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

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

Shon K, Kim Y, Colnago L, Opella SJ. 1991. NMR studies of the structure and dynamics of membrane bound bacteriophage Pf1 coat protein. *Science 252*:1303–1305.

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

Song HY, Cohen FS, Cramer WA. 1991. Membrane topography of ColE1 gene products: The hydrophobic anchor of the colicin E1 channel is a helical hairpin. *J Bacteriol 173*:2927–2934.

Wiener MC, Freymann D, Stroud RM, Ghosh P. 1996. Crystal structure of colicin Ia. *Nature 385*:461–464.

Wu CH, Ramamoorthy A, Opella SJ. 1994. High-resolution heteronuclear dipolar solid-state NMR spectroscopy. *J Magn Res 109*:270–272.

Zakharov SD, Heymann JB, Zhang YL, Cramer, WA. 1996. Characterization of membrane binding of the colicin E1 channel domain: Maximum activity is associated with an intermediate strength of the surface electrostatic interaction. *Biophys J 70*:2774–2783.

Zakharov SD, Venyaminov SY, Prendergast FG, Cramer WA. 1997. Structure analysis of the C-terminal channel domain of colicin E1 in its solution and membrane-bound states. *Biophys J 72*:A230.

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