Toward Elucidating the Membrane Topology of Helix Two of the Colicin E1 Channel Domain*

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The membrane-bound closed state of the colicin E1 channel domain was investigated by site-directed fluorescence labeling using a bimane fluorophore attached to each single cysteine residue within helix 2 of each mutant protein. The fluorescence properties of the bimane fluorophore were measured for the membrane-associated form of the closed channel and included fluorescence emission maximum, fluorescence anisotropy, apparent polarity, surface accessibility, and membrane bilayer penetration depth. The fluorescence data show that helix 2 is an amphipathic α-helix that is situated parallel to the membrane surface, but it is less deeply embedded within the bilayer interface region than is helix 1 in the closed channel. A least squares fit of the various data sets to a harmonic wave function indicated that the periodicity and angular frequency for helix 2 in the membrane-bound state are typical for an amphipathic α-helix (3.8 ± 0.1 residues per turn and 94 ± 4°, respectively) that is located at an interfacial region of a membrane bilayer. Dual quencher analysis also revealed that helix 2 is peripherally membrane associated, with one face of the helix dipping into the interfacial region of the lipid bilayer and the other face projecting outwardly into the aqueous solvent. Finally, our data show that helices 1 and 2 remain independent helices upon membrane association with a short connector link (Tyr363–Gly364) and that short amphipathic α-helices participate in the formation of a lipid-dependent, toroidal pore for this colicin.

Colicin E1 is a member of a large family of plasmid-encoded antimicrobial protein toxins secreted by Escherichia coli. They are produced by the bacteria in response to a variety of assaults, including DNA damage, anaerobiosis, catabolite repression, and nutrient depletion (1), targeting susceptible E. coli and similar bacteria. Colicins can be grouped into the following three categories based on their mode of action: (i) membrane depolarization via formation of ion-conducting channels (2), (ii) inhibition of protein (3) or peptidoglycan synthesis (4); and (iii) DNA degradation (5). Because of their ability to cross the Gram-negative bacterial membrane, colicins have become a model for studying bacterial protein import (6) as well as protein unfolding and folding (7, 8), membrane insertion (9, 10), and pore formation (11).

Colicin E1 belongs to the ion channel-forming group of colicins, which also includes colicins A, B, Ia, Ib, N, and K (12, 13). The structural organization of these pore-forming colicins includes three domains as follows: receptor binding, translocation, and catalytic domains. The receptor binding domain initiates entry of the toxin into the target cells (14) by binding with the BtuB, or vitamin B12, receptor on the bacterial outer membrane (15, 16). Next, the translocation domain associates with the trimeric β-barrel TolC outer membrane receptor, through which the unfolded translocation and catalytic domains of the colicin are transported into the periplasmic space (16). The catalytic domain subsequently refolds into an insertion-complement conformation, and the protein binds the inner membrane and, in a series of kinetically defined steps, the channel protein sequentially unfolds, binds, and spontaneously inserts into the membrane in a precursor state to the open channel (7, 17–19). The channel is opened upon imposition of a trans-negative membrane potential (20), and the newly created pore causes depolarization of the cytoplasmic membrane through the escape of cellular ions such as Na⁺, K⁺, and H⁺. As the bacterial cell tries to equilibrate the ion concentration using the Na⁺/K⁺-ATPase, cellular ATP reserves are rapidly depleted and cannot be replenished sufficiently, and cell death ensues (21).

A crystal structure of the soluble channel domain of colicin E1 was determined to a resolution of 2.5 Å (21). The data revealed a globular protein composed of 10 amphipathic α-helices similar to the previously published structure of colicin A (22). Subsequently, the whole or catalytic domain structure of pore-forming colicins Ia (23), N (24), and B (25) have been solved, and predictably they are all structurally similar. These structures suggest that a key to their pore-forming ability are the two distinct hydrophobic helices that create a membrane-spanning hairpin upon bilayer association (17, 26, 27). Interestingly, this feature of the colicin structure is also seen in the mammalian intracellular apoptotic regulators Bcl-2 (28), Bcl-XL (29), Bax (30), and Bid (31), proteins known to create pores upon association with the mitochondrial or endoplasmic reticulum membranes.

A plethora of approaches have been employed over the years to derive information about the membrane-associated closed channel topology of the catalytic domain of colicin E1. Solid state NMR was used to identify the existence of trans-membr-
brane and in-plane α-helices of membrane-bound colicin E1 (18), whereas circular dichroism, Fourier transform infrared spectroscopy, fluorescence resonance energy transfer, and differential scanning calorimetry were used to study membrane association and determine the α-helical and β-sheet content (32). Using site-directed mutagenesis to engineer conveniently located Trp residues, our group has estimated the depth of colicin E1 helical segments upon binding to the membrane using fluorescence quenching, fluorescence resonance energy transfer, and red-edge excitation shift analysis (33–35). These varied studies imply that the hydrophobic helices 8 and 9 insert into the bacterial inner membrane, whereas the remaining amphipathic helices lie on the membrane surface with considerable interaction with the aqueous medium. The exact orientation of the helices and the lipid and protein contacts made by each residue upon membrane binding are currently under investigation. Using electron paramagnetic resonance, the mobility and accessibility of nitroxide-labeled cysteine side chains generated one-at-a-time from residues 402–424 (which encompasses helix 4) showed a solvent-exposed and a lipid-exposed face of the amphipathic helix, suggesting a possible transmembrane orientation of this helix upon bilayer binding (36). Recently we reported on the membrane topology of helix 1 of colicin E1 in the closed channel state and found that this helix retains its α-helical structure and helix length even when bound at the surface of large unilamellar vesicles (37).

Herein we continue our investigation of the membrane-bound topology of the helices within the closed state of the colicin E1 channel with a study of the membrane-bound disposition of helix 2 (Glu^365–Ser^378). Using site-directed fluorescence labeling of a series of cysteine mutants coupled with rigorous analysis using various fluorescence techniques, we found that helix 2 is an amphipathic α-helix. However, it is less tightly appressed to the membrane surface than helix 1, and its membrane-bound length likely extends from Glu^365–Ser^378.

**EXPERIMENTAL PROCEDURES**

All chemicals, unless otherwise stated, were purchased from Sigma. All steady-state fluorescence measurements were collected using a PTI-Alphascan-2 spectrofluorometer (Photon Technologies Inc, South Brunswick, NJ) equipped with a thermostated cell holder. All measurements are reported as the mean ± S.D. and were performed at least in triplicate.

**Mutagenesis, Protein Purification, and Monobromobimane Labeling**—Cysteine-scanning mutagenesis, in which each amino acid residue from Glu^365 to Gly^380 of P190H6 was individually replaced with a cysteine, was performed using the Stratagene (La Jolla, CA) Quikchange™ mutagenesis kit. Plasmid DNA was purified using the High Pure Plasmid™ isolation kit from Roche Diagnostics. Wild type P190H6, P190H6/C505A (Cys-less wild type), and Cys mutant plasmids were prepared and purified from transformed le^-2

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2 The abbreviations used are: P190H6, colicin E1 190-residue channel domain; N-terminal 6 histidine tag; bimane-Cys, bimane-labeled N-acetyl-cysteine; DMG, dimethylglycine acid; LUVs, large unilamellar vesicles; mBBr, monobromobimane; Q-ratio, the ratio of quenching by 10-DN to that by KI; SASA, solvent-accessible surface area; WT, wild type; 10-DN, 10-doxyl/monadecane.
polarized light. Using the $I_{\text{VV}}$ and $I_{\text{VH}}$ fluorescence intensities, the anisotropy ($r$) was calculated as shown in Equation 1,

$$r = \frac{I_{\text{VV}} - G_{\text{H}}}{I_{\text{VV}} + 2G_{\text{H}}} \quad (\text{Eq. 1})$$

The “G” instrumental factor, measured as $I_{\text{IVV}}/I_{\text{IVH}}$, was determined from the intensities of the vertically ($I_{\text{IVV}}$) and horizontally ($I_{\text{IVH}}$) polarized emitted light from horizontally polarized excitation light. For all measurements, the excitation was set at 381 nm (4 nm slit-width), and emission was collected at 470 nm (10 nm slit-width) with a signal integration time of 30 s. Each anisotropy value is the mean of three determinations. A solvent blank (buffer or LUVs in buffer) was subtracted from each intensity reading prior to the calculation of the anisotropy value as described previously (37).

**Dual Quenching Analysis**—Depth-dependent quenching of membrane-bound bimane-labeled mutant proteins was performed as described previously (41). To measure iodide quenching ($F_{\text{Kl}}$), the fluorescence of samples was measured on a Spex Tau-2 Fluorolog spectrofluorimeter (Jobin Yvon Inc., Edison, NJ) in ratio mode using semi-micro quartz cuvettes (excitation path length 10 mm, emission path length 4 mm) containing 100 $\mu$M LUVs and 7.5 $\mu$g of protein or LUVs only (background). $F_{\text{Kl}}$ was determined 5 min after the addition of a 50-$\mu$L aliquot of an aqueous solution from a 1.7 mM KI and 0.85 mM Na$_2$S$_2$O$_3$ stock solution. The fluorescence values after KI addition were corrected for dilution before quenching was calculated. The excitation wavelength was set at 375 nm while observing the emission intensity at 467 nm. The excitation and emission slit-widths were 2.5 and 5.0 nm, respectively. To measure the efficiency of 10-doxynonadecane (10-DN) quenching, membrane-bound protein or vesicles lacking protein were prepared as described above except that the 10-DN quencher-containing LUVs contained 10 mol % of 10-DN. After preparation, all the samples were allowed to equilibrate for 30 min at 24 °C before measurement of initial fluorescence.

**Calculation of the Iodide to 10-DN Quenching Ratio (Q-Ratio)**—The ratio of quenching by 10-DN to that by KI (Q-ratio) was used to determine bimane depth in lipid bilayers. The Q-ratio was calculated from Equation 2,

$$Q\text{-ratio} = \frac{(F_{r}/F_{\text{10-DN}}) - 1}{(F_{r}/F_{\text{Kl}}) - 1} \quad (\text{Eq. 2})$$

where $F_r$ is the fluorescence of a sample lacking quencher, and $F_{\text{Kl}}$ and $F_{\text{10-DN}}$ are the fluorescence intensities in the presence of KI or 10-DN, respectively.

**Sensitivity of the Fluorescence Parameters of Bimane to Solvent Polarity**—The sensitivity of the bimane fluorescence to solvent polarity was assessed using N-acetylcyesteine conjugated with bimane (bimane-Cys) as a probe. Bimane-Cys was produced by reacting mBBr with 10-fold molar excess of N-acetylcysteine in 100 mM NH$_4$HCO$_3$ buffer, pH 8.1, for 1 h. The reaction mixture was lyophilized overnight and subsequently resuspended in dioxane:water mixtures of 0–100% (v/v) dioxane. The fluorescence emission and lifetime of bimane-Cys (2 $\mu$M bimane-Cys) samples in dioxane:water mixtures of different dielectric constants ($\epsilon = 2.3–80$) were determined.

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**Predicting Secondary Structure from Fluorescence Parameters**—The secondary structure elements were predicted from the observed fluorescence parameters using a method adopted from Cornette et al. (42). In brief, the periodicity and the angular frequency of the observed fluorescence parameters were obtained through a least squares fitting approach using the harmonic wave function shown in Equation 3,

$$y = a \cdot \sin \left(2\pi \left(\frac{x + b}{p}\right)\right) + c \quad (\text{Eq. 3})$$

where $a$ is the amplitude; $b$ is the phase; $p$ is the period, and $c$ is an offset value. All calculations were made using an Excel 2002 template spreadsheet developed for us by Dr. Uwe Oehler.

**RESULTS**

**Structural and Functional Analysis**—The primary amino acid sequence and ribbon diagram of the crystal structure (21) of the channel peptide of colicin E1 (P190) can be seen in Fig. 1. The designation “P190H$_6$” refers to the N-terminal His$_6$-tagged, 190-amino acid peptide of colicin E1 that encodes the active channel domain. In Fig. 1A, the sequence of the protein corresponding to helix 2 that was subjected to Cys-scanning mutagenesis is indicated by the downward facing brace. The soluble structure of the domain (Fig. 1B) is arranged in three layers as follows: (a) helices 1, 2, and 10; (b) helices 5, 8, and 9; and (c) helices 3, 4, 6, and 7. The locations of the Cys-substituted residues are also highlighted in Fig. 1B by dark spheres that correspond to the C-$\alpha$ carbon of each residue.

The labeling efficiencies of most of the Cys mutants ranged from 60 to 110% indicating the newly incorporated cysteine residues were labeled as expected. However, problems were encountered with A371C (9% labeling efficiency) and K377C (200% labeling efficiency).

The WT P190H$_6$ protein contains a single Cys residue at position 505 within helix 9 of the structure. This cysteine residue is extremely buried and does not normally react with electrophilic reagents used to covalently tether a fluorophore to an engineered Cys residue at the surface of a mutant protein. However, Cys$_{505}$ labeling would be a problem for the A371C and K377C mutants. In the case of A371C, even a small amount of Cys$_{505}$ labeling would contribute to the bimane signal, and in the case of K377C, the extra labeling suggests Cys$_{505}$ probably gets labeled because of some conformational perturbation.

The most likely explanation for the behavior of the K377C mutant is that the mutation of the long lysine residue to a more hydrophilic reagents used to covalently tether a fluorophore to an engineered Cys residue at the surface of a mutant protein. However, Cys$_{505}$ labeling would be a problem for the A371C and K377C mutants. In the case of A371C, even a small amount of Cys$_{505}$ labeling would contribute to the bimane signal, and in the case of K377C, the extra labeling suggests Cys$_{505}$ probably gets labeled because of some conformational perturbation.

The low labeling efficiency of the A371C mutant could be ascribed to the fact that the relatively hydrophobic Ala$_{371}$ residue is located in the middle of helix 2 facing toward the core hydrophobic hairpin helices of the channel peptide and is therefore inaccessible to the labeling reagent. However, bimane labeling experiments of A371C/C505A even in the presence of
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FIGURE 1. Schematic representation of the channel domain of colicin E1. A, the primary sequence and the secondary structure of the channel-forming domain are shown as Tr and Th, respectively. B, the ribbon topology diagram of the 2.5 Å crystal structure of the P190 peptide (21). The overall architecture of the structure consists of 10 helices, a hydrophobic helical hairpin (8-9, black) surrounded by eight amphipathic helices that are arranged in a three-layer sandwich helical bundle as follows: layer A, helices 1, 2, and 10; layer B, helices 8, 9, and 5; layer C, helices 3, 4, 6, and 7 (21). The location of the cysteine mutation sites are highlighted with residues corresponding to the Cys substitutions. The tryptic and thermolytic digestion sites of the channel-forming domain are shown as Cys substitutions. The tryptic and thermolytic digestion sites of the channel-forming domain are shown as Cys substitutions. The tryptic and thermolytic digestion sites of the channel-forming domain are shown as Cys substitutions.

8 M urea did not improve the labeling efficiency (data not shown).

Whatever, the origin of these problems, to circumvent them, a Cys-less P190H,e (P190H,e/C505A) was used as a background template. As expected, the use of the K377C/C505A double mutant returned the bimane labeling efficiency to normal levels (83%) for this mutant.

To determine the folding and functional properties of all mutants, the intrinsic Trp fluorescence and in vitro channel activities of each protein, with or without the bimane label, were determined. The intrinsc Trp fluorescence measurement provides an assessment of the folded integrity of the protein under investigation; a Trp \( \lambda_{\text{em}}^{\text{max}} \) value near 324 nm for colicin E1 indicates properly folded protein, whereas more red-shifted values indicate perturbation of the folded structure (at a Trp \( \lambda_{\text{em}}^{\text{max}} \) value near 350 nm, the protein is considered fully denatured (43)). As shown in Table 1, the structural and functional integrities of most mutant proteins were comparable with the WT (P190H,e) or Cys-less template (C505A) as per their Trp \( \lambda_{\text{em}}^{\text{max}} \) values and their relative in vitro channel activities. One mutant, M370C, had a slightly red-shifted Trp \( \lambda_{\text{em}}^{\text{max}} \) (329 nm), indicating a degree of perturbation; however, upon bimane labeling, it seems to have reacquired the native folded structure (Trp \( \lambda_{\text{em}}^{\text{max}} \), 325 nm). Two other mutants, Y367C and L374C, displayed a more significant Trp \( \lambda_{\text{em}}^{\text{max}} \) red shift to 333 and 332 nm, respectively, suggesting that these mutant proteins were somewhat destabilized by Cys substitution. Not surprisingly, these two mutants did not express high quantities of protein, and therefore protein purification was less successful (data not shown). In the case of Y367C, analysis of the crystal structure of the channel peptide revealed that the mutation likely caused the loss of a critical hydrogen bond with Thr\(^{501}\) of helix 9. This most likely “loosened” the tertiary structure at this point causing local unfolding. The underlying cause of the red-shifted Trp \( \lambda_{\text{em}}^{\text{max}} \) seen for L374C was not immediately obvious; however, a general loosening of the structure induced by Cys substitution is likely the explanation. However, both of these mutant channel proteins were active and exhibited good in vitro channel activities (Table 1).

Solution Bimane Fluorescence Emission and Solvent-accessible Surface Area of Cys-probed Sites—Fig. 2A shows the bimane \( \lambda_{\text{em}}^{\text{max}} \) values for the Cys-labeled sites in helix 2 of the soluble channel domain. In the soluble form, most of the mutants showed bimane \( \lambda_{\text{em}}^{\text{max}} \) values \( \approx 470 \text{ nm} \). These mutant proteins correspond to mostly polar and charged residue substitutions, E365C, Y367C, S368C, K369C, Q372C, E373C, D376C, K377C/C505A, S378C, K379C, and G380C. The corresponding SASA values of the substituted sites also showed nearly consistent accessible surface area values (SASA \( >50 \text{ Å}^2 \); Fig. 3, solid line), a value that is generally accepted for surface-exposed sites within a soluble protein (44). In contrast to the surface-exposed mutations, the remaining mutant proteins, M370C, A371C/
C505A, L374C, and A375C, showed relatively blue-shifted bimane/em(max) values (455–469 nm) (Fig. 2A), and the SASA values of the Cys sites of these mutants showed correspondingly lower surface-accessible areas (0–5.23 Å²) indicative of buried sites within the soluble channel domain. An exception to this correlation of SASA with the em(max) was observed for Y367C, S368C, S378C, and G380C mutants. According to the predicted SASA values, the Cys-labeled sites of these mutants have rather low accessible surface areas (0–2.99 Å²) and hence would be expected to be buried sites within the protein. However, the observed em(max) values of these mutants (470–479 nm) are consistent with more surface-exposed sites. This discrepancy can be explained if one takes a closer look at the location of substitution sites in the crystal structure of the colicin E1 channel domain (Fig. 1B). Tyr367 and Ser368 are situated at an extremely tight junction because of the small, tight turn between helix 1 and helix 2. It is possible that a mutation and subsequent labeling at Tyr367 and Ser368 may elicit localized structural perturbation, rendering the tethered bimane fluorophore more exposed than the native residues (Tyr or Ser). In fact, the Trp/em(max) of Y367C (333 nm) indicates some structural perturbation of this mutant in comparison with the WT (Trp/em(max) = 324 nm) as a consequence of the mutation and labeling procedures, and this mutant has the lowest channel activity of all those studied in helix 2 (60% of WT activity, see Table 1). For G380C, closer inspection of the crystal structure indeed confirms that the Gly380 residue is surface-exposed, and the predicted low accessible surface area is attributed to the limitation of the algorithm used in determining the SASA of small side chain moieties such as Gly (H atom) (40). Despite these minor limitations, the observed bimane/em(max) for most mutant proteins in aqueous solution appear to correlate well with the solvent accessibility of the probed sites for the solution structure of the colicin E1 channel domain. Most of residues with high SASA (>50 Å²) showed consistently higher em(max) values (>470 nm) and vice versa. Such observations are con-

![Figure 2](image2.png)

**FIGURE 2.** The fluorescence emission maximum (em(max)) of bimane-labeled Cys mutants of helix 2 of the colicin E1 channel domain. A, the bimane em(max) values of the Cys mutants of the soluble channel domain fitted through nonlinear least squares analysis to a harmonic wave function (r² = 0.80). B, the bimane em(max) values of the membrane-associated Cys mutants fitted through nonlinear least squares analysis to a harmonic wave function (r² = 0.81). The data points are joined by a dashed line, and the harmonic function fit to the data is shown as a solid line (periodic function). Average values and standard deviations for experiments completed in triplicate are shown.

![Figure 3](image3.png)

**FIGURE 3.** Side chain SASA and solvent accessibility of the Cys-substituted residues within helix 2 of the soluble channel domain. A, solvent-accessible surface areas of substituted side chains were calculated from the coordinates of the crystal structure of soluble P190 peptide using 1.4 Å probe as described under the “Experimental Procedures” (Protein Data Bank entry 2I88). B, the solvent accessibility of each residue was calculated as the ratio of the side chain surface area as estimated from the crystal structure and the expected theoretical value of each residue in the random coil tripeptide, Gly-X-Gly. Residues with solvent accessibility ratios above 50% (top horizontal line) are considered to be surface-exposed, whereas residues with ratios ≤20% (bottom horizontal line) are buried. The calculated SASA data were fitted through nonlinear least squares analysis to a harmonic wave function (r² = 0.91 and 0.92, respectively). The data points are joined by a dashed line, and the harmonic function fit to the data is shown as a solid line (periodic function).
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sistent with our earlier characterization of helix 1 of the channel peptide (37).

Membrane-bound Bimane Fluorescence Emission and the Topology of the Cys Sites—Fig. 2B shows the bimane λ_em(max) of membrane-bound Cys mutant proteins. In comparison to Fig. 2A, the overall shape and profile of the λ_em(max) values of this helix do not differ from that of the soluble state, indicating that the amphipathic α-helical nature of this segment of the channel protein was not significantly affected upon binding to the membrane surface. Overall, the observed λ_em(max) values of the membrane-bound protein showed slightly blue-shifted λ_em(max) values in comparison to the solution state of the corresponding mutants. As shown previously for helix 1, this shift is consistent with overall changes in the polarity of the local environment of the bound peptide upon membrane association (37). On the basis of the general λ_em(max) distributions, the membrane-bound mutants within helix 2 can be grouped into three categories as follows: 1) mutant proteins with red-shifted λ_em(max) (≥470 nm), E365C, S368C, K369C, Q372C, E373C, D376C, K379C, and G380C; 2) mutant proteins with intermediate λ_em(max) values (468–469 nm), Y367C, L374C, and K377C/C505A; and 3) mutant proteins with blue-shifted λ_em(max) (<468 nm), M370C, A371C/C505A, A375C, and S378C. Group 1 mutants, corresponding to largely polar and charged residues substituted with Cys, form the surface-exposed face of helix 1 in the soluble structure (Fig. 1B). Group 3 mutants, consisting of relatively nonpolar or polar uncharged residues replaced with Cys, make up the more buried face of this helix. Some of the residues in the intermediate group, such as Leu374, will likely be part of the hydrophobic face of the helix, whereas Tyr367 and Lys377 would be expected to interact with the aqueous solvent given the length of their side chain moieties, but a Cys substitution of the latter residues may lead to an interfacial location for the bimane chromophore.

To further characterize the nature of the local environment surrounding the Cys-scanned sites within helix 2 of the membrane-bound channel domain, the apparent polarity corresponding to observed λ_em(max) values were calculated using the equation λ_em(max) = 0.33 (nm/e) × D + 452.8 nm, where D is the dielectric constant of the bulk solvent (e). This relationship was determined in our previous study (37) with a bimane-N-acetyl-Cys standard and a dioxane-water solvent system; this polarity-calibration approach was found to be a reliable indicator of the apparent polarity of the local environment for the bimane chromophore. The calculated apparent polarities for group 1 mutants ranged between ε = 69 and 53; these values are consistent with highly aqueous, solvent-accessible environments. As for groups 2 and 3, the calculated apparent polarities ranged from ε = 48–50 and ε = 13–41, respectively. Although the latter two groups reside in a relatively more nonpolar environment than the group 1 mutants, the calculated apparent polarities of these two groups are nonetheless outside the range generally accepted for even the interfacial layer of the membrane (ε = 10–25). Only two mutants, A371C/C505A and S378C, showed apparent polarity values (ε = 13 and 22, respectively) that are within the range generally ascribed to the interfacial layer of the membrane.
sites within unstructured regions (random coil). The observed probe mobility for most of the mutant proteins (Fig. 4A) in solution correlated well with the available surface area for the side chains of the probed sites, as per the calculated SASA from the solution crystal structure (Fig. 3) as well as the \( \lambda_{em(max)} \) (Fig. 2A). Mutant proteins corresponding to sites predicted to have large accessible surface areas and shown to possess high \( \lambda_{em(max)} \) values also showed higher probe mobility relative to the buried sites. E365C, K369C, E373C, D376C, K377C/C505A, K379C, and G380C, all of which showed \( \lambda_{em(max)} \) values greater than 470 nm in the soluble state and that correspond to sites with greater than 50 Å\(^2\) accessible surface area, also exhibited higher probe mobility (7.9–10.5).

In contrast, L374C, A375C, and S378C, which correspond to sites with less than 10 Å\(^2\) accessible surface areas and are thus buried in the core of the protein, showed lower probe mobility (5.8–6.4). It should also be noted that the observed probe mobility in the soluble state of mutant proteins of the N-terminal region of helix 2 do not agree well with the estimated accessible surface area of the substituted side chains (Fig. 3) and the observed \( \lambda_{em(max)} \) of these proteins in solution. For instance, M370C and A371C/C505A show unusually high probe mobility (10.5 and 7.4, respectively) given the predicted accessible area of their substituted side chains (5.2 and 0.5 Å\(^2\)), respectively and the corresponding \( \lambda_{em(max)} \) values (469 and 455 nm), both of which suggest the labeled sites to be facing the interior of the proteins. Similar discrepancies are also observed for Y367C, S368C, and Q372C, which also show lower than expected probe mobility (Fig. 4A). It is likely that these discrepancies may reflect structural alterations to the tight packing of this region of the protein (Fig. 1B) as a consequence of the tethered bimane and the introduced mutations. Interestingly, despite some localized discrepancies for the observed fluorescence parameters with the crystal structure data, the overall profiles of both \( \lambda_{em(max)} \) and the probe mobility data show that the \( \alpha \)-helical structure of helix 2 of the soluble channel domain remains intact after Cys mutation and also after bimane attachment.

The observed probe mobility for membrane-bound helix 2 mutant proteins (Fig. 4B) correlated well with their \( \lambda_{em(max)} \) values (Fig. 2B). Labeled sites predicted to form the hydrophobic side of the helix that faces the membrane surface in the membrane-bound state, as per \( \lambda_{em(max)} \) and estimated apparent polarities, showed equally low probe mobility values (4.7–5.4). Similarly, labeled sites shown to form the surface-exposed face of helix 2 also displayed relatively higher probe mobility values (5.9–7.9). Comparison of the overall profiles of the probe mobility values of the membrane-bound state and the soluble state of the labeled sites, with the exception of the N-terminal region for the above described reasons, reveal a similar pattern and periodicity, consistent with an amphipathic \( \alpha \)-helical secondary structure conformation for helix 2 in both the soluble and membrane-bound states.

**Dual Quenching Analysis of the Membrane-bound Depth of P190HC\(_6\) Helix 2**—To assess the relative bilayer depth of helix 2 residues, a dual quenching fluorescence method that measures the depth of fluorescent groups in membranes was used (37, 41, 45, 46). This method exploits the quenching \(((F_0/F_q) - 1)\) of the fluorescent groups at each site by two quencher species (KI and 10-DN) that possess differential solubility with respect to the aqueous solvent and the membrane bilayer \((F_0\) and \(F_q\) represent the fluorescent intensity of the bimane fluorophore in the absence and presence of quencher, respectively). In these experiments, membrane-inserted fluorescent groups are strongly quenched by the membrane-embedded quencher (10-DN), giving a high value of \(((F_0/F_{10-DN}) - 1)\). In contrast, surface-exposed fluorescent groups, including bimane, are strongly quenched by the aqueous quencher (KI), giving a high value of \(((F_0/F_{KI}) - 1)\). Given that the accessibility to any single quencher could be affected by the local protein conformation, the quenching ratio (Q-ratio; \(((F_0/F_{10-DN}) - 1)/(F_0/F_{KI}) - 1)\) of the nonpolar and polar quencher pair best describes the relative depth of the probed site. A low Q-ratio indicates that the bimane probe is quenched more by KI and less by 10-DN and would be expected to be localized outside the membrane environment.

Similarly, a high Q-ratio indicates significant membrane depth penetration of the probe into the bilayer and therefore shows less quenching by the water-soluble KI and more by 10-DN (41, 45, 46). Fig. 5 shows the dual quenching analysis data for both helix 1 and helix 2 residues (37). The Q-ratio data for helix 2 residues (Fig. 5, right-hand side) are in good agreement with the data obtained by bimane emission and probe mobility data, showing that this helix is lying close to the membrane-water interface with considerable exposure to the aqueous KI quencher and limited contact with the hydrophobic 10-DN quencher. The tethered bimanes of all of the mutant proteins of helix 2 are quenched to a much greater degree by the water-soluble quencher, KI (Fig. 5A, gray bars), than the membrane-embedded quencher, 10-DN, under the conditions chosen for the experiments (Fig. 5A, black bars). The only proteins quenched by 10-DN at an appreciable level are M370C, A371C/C505A, L374C A375C, and S378C, all of which represent sites within helix 2 that would be expected to face the membrane surface based upon the \( \lambda_{em(max)} \) and probe mobility data (Fig. 2B and Fig. 4B).

**Predicting Secondary Structure from Fluorescence Parameters**—To determine the secondary structure of helix 2 of the membrane-bound channel domain, we used the method of Cornette et al. (42) to analyze the periodic variation of the observed fluorescence parameters with the residue number within this helix as described earlier for helix 1 (37). Shown in Table 2 is the summary of the nonlinear least squares harmonic wave function analysis of the observed fluorescence parameters of helix 2 for the both the soluble and membrane-bound states of the channel domain. For the soluble state, the fitting of the observed fluorescence parameters and the SASA data obtained from the crystal structure confirmed the amphipathic \( \alpha \)-helical structure of this segment of the protein. The calculated residue per turn periodicity (\( \rho \)) of both observed parameters (\( \lambda_{em(max)} \) and probe mobility) and those obtained from the crystal structure show a consistent value of 3.7 ± 0.1, which is in agreement with the periodicity of an amphipathic \( \alpha \)-helix (40, 44). The phase of the fitted sine wave function for the \( \lambda_{em(max)} \) and SASA data show comparable values, indicating that the orientation of the two faces of helix 2 did not change significantly upon Cys

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Membrane-bound Topology of Helix 2 of Colicin E1

replacement and bimane labeling. However, the reduced periodicity and phase values of the probe mobility data in comparison to the SASA data may be an indication of slight alteration in tertiary contacts without concomitant secondary structure changes, especially at the N-terminal region of helix 2, induced by the Cys scanning method. The analysis of the amphipathic \( \alpha \)-helical property of this segment of protein in the membrane-associated state showed that helix 2 maintains its amphipathic character with an average \( p \) value of 3.8 ± 0.1 residues per turn. The phase property of this periodicity (228–233°) and residues mapped to form the helix (365–379) are in agreement with those obtained from the analysis of the SASA data for helix 2 in the soluble state.

DISCUSSION

Previously, we showed that helix 1 within the colicin E1 channel domain maintains its amphipathic \( \alpha \)-helical structure upon binding to the membrane surface in the closed channel state (37) and that the soluble helix consists of residues 350–362, whereas the membrane-bound helix consists of residues 347–362 with the Tyr\(^{363} \)-Gly\(^{364} \) sequence serving as a helix breaker between helices 1 and 2. The data pertaining to helix 2 herein demonstrate that this helix is also relatively insensitive to perturbation (Table 1). Furthermore, channel activity is also insensitive to inactivation by Cys replacement mutagenesis and subsequent bimane labeling (37) (Table 1). The greatest reduction in the \textit{in vitro} channel activity for the helix 2 mutant proteins was only 40% (G380C mutant, Table 1), which supports previous mutagenesis studies that demonstrated the robust nature of the colicin E1 channel domain and its insensitivity to single point mutations (47–51).

The site-directed fluorescence labeling data presented herein provide compelling evidence for the amphipathic nature of helix 2 within the channel domain when it is bound to the membrane bilayer in the closed channel state (Fig. 6). It is clear from the harmonic wave function analysis of both helices 1 and 2 that the periodicities and angular frequencies of these helices do not change significantly upon binding to the membrane surface. In a similar fashion observed for helix 1, helix 2 also is appressed to the membrane surface with its nonpolar face bathing the hydrocarbon portion of the bilayer. However, the \( \lambda_{em(max)} \) and apparent polarity data indicate that helix 2 is less tightly appressed to the bilayer surface than helix 1 and is overall more exposed to the aqueous solvent. Importantly, the connection between helix 1 and 2, Tyr\(^{363} \)-Gly\(^{364} \) in the membrane-bound channel domain, is not helical and therefore points to the individual nature of helices 1 and 2 in the closed channel state, \( i.e. \) that helices 1 and 2 do not join to form a longer \( \alpha \)-helix lying on the membrane surface (Fig. 6) and that likely this linker

![FIGURE 5. Comparison of the relative membrane bilayer depth for bimane-labeled P190H\(_6\) helix 1 and helix 2 mutant peptides incorporated into lipid vesicles. A, histogram showing the extent of bimane fluorescence quenching by KI (gray bar) and by 10-DN (black bar). We estimate that values within 0.1–10 show significant quenching with 95% confidence as judged from simulated data of the quenching function. B, plot of the quenching ratio \((F_0/F_{10-DN}) - 1)/(F_0/F_{KI}) - 1\) (Q-ratios) for tethered bimane. Average values and standard deviations for experiments completed in triplicate are shown.](image)

| Parameters | Soluble | Membrane-associated |
|------------|---------|---------------------|
|            | \( p^a \) | Phase\(^b \) | Residues\(^c \) | \( p^a \) | Phase\(^b \) | Residues\(^c \) |
| SASA       | 3.7 (97.0°)\(^d \) | 230 | 367–378 | 3.8 (95.4°) | 228 | 367–378 |
| \( \lambda_{em(max)} \) | 3.8 (96.5°) | 228 | 367–378 | 3.7 (96.5°) | 233 | 367–378 |
| Mobility   | 3.6 (98.5°) | 183 | 367–378 | 4.0 (90.0°) | 228 | 368–379 |
| Q-ratio    |         |         |         | 3.8 ± 0.1 (94.2 ± 4.0°) |  |

\( a \) The residue-per-turn (rpt) periodicity of the parameters is shown.

\( b \) The phase of the fitted harmonic sine wave is shown.

\( c \) Residues were used in the fitting process.

\( d \) The angular frequency of the periodicity is shown.
region may provide a hinge function in the transition to the helices 1 and 2.

FIGURE 6. Schematic representation of membrane-bound topology of helices 1 and 2. Shown in the top panel is the ribbon diagram with residue side chains of helices 1 and 2 oriented with their nonpolar faces toward the hydrocarbon milieu of the bilayer and their polar faces toward the aqueous medium. The bottom panel shows the relative membrane-bound topology of both helices. Helix 1 lies on the surface of the membrane with its nonpolar face bathing the hydrocarbon portion of the bilayer, whereas helix 2 is not as tightly appressed to the membrane surface but is more solvent-exposed with its nonpolar face pressed against the head group region of the phospholipid bilayer with a few residues extending into the interfacial layer. Also shown in the bottom panel are the accepted dielectric constants (ε) of the interfacial layer (IF; ε = 10–24) and the hydrophobic core of the acyl chain (HC, ε = 2–4).

The harmonic wave function analysis of our fluorescence anisotropy data for helix 2 (Fig. 2B and Fig. 4B; Table 2), providing convincing evidence that both helices 1 and 2 are bound to the surface of the membrane in a conformation parallel to the bilayer surface. Fig. 6 is a simple model of both helices in the closed channel state of colicin E1 that shows the disposition of these amphipathic helices and the deeper insertion within the membrane bilayer of helix 1 compared with helix 2. The shallow nature of helix 2 corroborates previous proteolysis data of the membrane-bound colicin E1 channel domain that indicated the relatively accessibility of helix 2 to protease digestion (54).

The structure of the open channel formed by colicins upon voltage imposition has been controversial. It is generally believed that the voltage-gated, open channel consists of a single polypeptide with an even number of transmembrane helices having the N and C termini lying on the cis side of the membrane and involves the movement of surprisingly large segments of the membrane-bound precursor across the membrane. Less controversial is the identity of the anchor domain of the channel, which is generally believed to consist of helices 8 and 9 of the soluble protein. However, the identity of the remaining transmembrane segments of the open channel is less clear, various models have been proposed, most of which involve the formation of at least two additional transmembrane helices (13, 34, 54–59). A number of these models include an extended helix formed by the expansion of helices 1 and 2 of the soluble structure to provide one of the pillars of the open channel structure. Although helix rearrangements are an important aspect of the mechanism of colicin E1 membrane association, insertion, and pre-channel state formation, our current data preclude the melding of helices 1 and 2 into an extended helix as a feature of the closed channel state for colicin E1 (37) (Fig. 6).

Our results contained herein for helix 2 and our previous data for helix 1 (37) favor a toroidal model for colicin E1 as proposed earlier by Cramer and co-workers (13, 58) that involves the formation of the open channel with relatively short amphipathic helices (15–16 residues) that employ phospholipids in an inverted micellar conformation in order to form a functional channel. Furthermore, it has been demonstrated recently that a much larger conductance channel (600 pS compared with 60 pS) may be formed in thicker bilayers and that colicin E1 may induce lipid “flip-flop” in such membranes (59). The toroidal lipid pore model has been used to describe the action of large toxins such as equinatoxin II (60) and sticholysin (61), antibiotic peptides (62, 63), and apoptotic pore-forming proteins Bax (64) and tBid (65). An important requirement for the toroidal model is the sensitivity of the pore-forming activity to membrane lipid curvature, where lipids with positive and negative spontaneous curvatures stimulate and inhibit pore formation, respectively. Cramer and co-workers (58) clearly demonstrated the sensitivity of colicin E1 pore-forming activity on membrane curvature lending support to the toroidal model for this bacteriocin and also that the nature of the channel is influenced by the thickness of the membrane bilayer (59).

The harmonic wave function analysis of our fluorescence data for helices 1 and 2 shows a break in the periodicity of all profiles beyond Lys362, both in the soluble and membrane-bound states of the colicin E1 channel domain. In the x-ray structure of the colicin E1 P190, Lys362 marks the C terminus of helix 1 and the start of a very tight turn (Tyr363 to Gly364) that separates helix 1 from helix 2 (37) (Fig. 1, A and B). Moreover, Gly364 is conserved in all helical colicins and is located in this short turn region that separates helix 1 and 2 in all known structures of channel-forming colicins. Additionally, our wave function analysis suggests that the C terminus of helix 2 is marked by Ser378 and is terminated by Lys379–Gly380. Accordingly, our data suggest that the helical boundaries of helices 1 and 2 remain unaffected upon binding to the membrane surface. Notably, our data refute earlier models that suggested the extension of parts of helix 1 and 2 into a single long amphipathic transmembrane helix as part of the open channel structure (53). These conclusions support the findings of Slatin et al. (57), who showed that segments of helix 1 from both colicin Ia and A are
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inserted into the membrane with the intervening loop forming a pivotal point between the transmembrane and the translocated segments of the open channel. In colicin A, this short loop is partially exposed to the trans side of the membrane, and the majority of helix 2 is translocated across the membrane. At present it is not clear as to how this translocation event contributes to the channel-gating mechanism. Obviously, there still exists considerable controversy as to the nature and structure of both the closed and open states of the pore-forming colicins. We will continue our pursuit of the membrane-bound topology of the closed state of colicin E1 through a residue-by-residue analysis using fluorescence-based methodology. It is anticipated that this approach, although tedious, will help to clarify some of the ambiguities and the enigma that currently define both the closed and open states of the pore-forming colicins.

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