Solvation Driven Conformational Transitions in the Second Transmembrane Domain of Mycobacteriophage Holin

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ABSTRACT:
Holins are pore-forming membrane proteins synthesized by lytic phages. The second transmembrane domain (TM2) of Mycobacteriophage D29 holin presents an Ala-and Gly-rich sequence, with a currently unknown structure and function. In this study, we present the spectroscopic characterization of synthetic TM2 in various solvents, detergents, and lipids. We find that TM2 adopts \( \alpha \)-helical conformation under conditions that promote intra-strand hydrogen bonding, such as organic solvents and detergent micelles. When we transfer the peptide to a well-hydrated environment, a polyproline II-like structure is obtained. Surprisingly, we find that the polyproline II-like conformation is retained in lipid vesicles. Based on our results, we present a putative role for TM2 in the process of pore formation by holin. © 2016 Wiley Periodicals, Inc. Biopolymers (Pept Sci) 108: 1–10, 2017.

Keywords: Mycobacteriophage holin; peptide helices; circular dichroism spectroscopy; transmembrane peptide; polyproline II structure

INTRODUCTION
Proteins execute a myriad of functions within the cell. They rely on structure and structural scaffolds that they adopt, to interact with other biomolecules and for enzymatic activity. Understanding proteins at the level of structure can provide information on the functional state of the molecule. Defined protein secondary structure elements form the building blocks of tertiary and quaternary assemblies, and they possess periodic repeats of backbone \( \phi - \psi \) values. The two major regular polypeptide conformations are \( \alpha \)-helices and \( \beta \)-sheets; additionally, the allowed region of the Ramachandran map also includes the \( 3_{10} \) helix, \( \pi \)-helix, left-handed \( \alpha \)-helix, \( \beta \)-turns, collagen triple helix, poly(Pro) I and II, and poly(Gly) I and II.\(^1\)

The restricted backbone \( \phi \) along with the ability of Pro to adopt both cis and trans conformations at the Xxx–Pro peptide bond favors Pro-rich sequences to adopt the poly(Pro) I (PPI) or poly(Pro) II (PPII) structures. Studies on unstructured segments of proteins have shown that the extended backbone conformation of PPII is usually abundant in these regions. The presence of PPII has a significant impact on the structural rigidity of these unstructured segments.\(^2\) Indeed, the PPII helix has appeared recently as the leading example for describing the conformation of unfolded polypeptides.\(^3,4\)
PPII exhibits left-handed chirality in nature, with restricted $\phi$ and $\psi$ angles of approximately $-78^\circ$ and $+149^\circ$, respectively. It can undergo slow conversion to the right-handed PPI structure when the solvent system is changed from water (or mild acids) to alcohols such as propanol and butanol. The PPII structure is involved in many roles, such as protein–protein interaction, $^5$ protein self-assembly, $^7$ protein–nonprotein interactions, $^9$ conferring three-dimensional structural integrity, $^{11}$ and also serves as an interdomain linker. $^{12}$

It is broadly accepted that PPII structures are observed in poly-$\text{L-}\text{Pro}$, poly-$\text{L-}\text{Glu}$, poly-$\text{L-}\text{Lys}$, and the collagen sequence. $^{4,13-15}$ However, segments that adopt PPII-like structures have been observed in short polypeptides and proteins. $^{16-19}$ PPII-like segments with a minimum of two residues and a maximum of 14 residues has been observed to date. $^{16,20}$ While Pro-rich regions show a preference for the PPII structure, host-guest based analysis using Pro-containing sequences as the host revealed that when Gln, Asp, Gly, and Ala (guest) are incorporated, the guest residues can adopt the PPII form. $^{21,22}$ This host–guest analysis using short peptides led to the understanding of how a single amino acid can play an important role in influencing a defined conformation.

In a recent finding, the groups of Kallenbach and Rose used a comprehensive analysis to assess how nonproline (Pro–) peptides can adopt PPII-like backbone $\phi$–$\psi$ values. $^{23-26}$ They highlighted the ability of Ala- and Gly-containing short peptides to form PPII–like structures. $^{24-29}$ Such Pro– PPII structures play important roles in proteins. For example, they form the interdomain linker in calcium binding domains, $^{2,30}$ structure present in antigen epitope which binds to MHC class II, $^{31}$ and are seen in protein–nucleic acid interactions. $^{32}$ The studies on Pro– peptides, therefore, have established the role of amino acid preferences in PPII.

The polypeptide sequence displays an overall preference to fold and attain the desired secondary structure; however, this final state can also be governed by environmental conditions. For example, solvent dehydration and rehydration, $^{26,33,34}$ pH, $^{22,35}$ ionic concentration, $^{35,36}$ and temperature $^{37,38}$ influence the PPI structure. In particular, the solvent conditions contribute considerably to the structural features of PPII, govern its conversion to PPII, and thereby promote the protein or polypeptide to attain stable conformation.

As the observation of a PPII helix is limited to short peptide segments (2–14 residues), due to the restricted $\phi$–$\psi$, amino acid propensities for such structures are also limited. A high intrinsic propensity is known for Gln, Ala, and Gly, apart from Pro, and only in soluble proteins or peptides. There are very few reports of a transmembrane peptide or protein sequence capable of adopting a PPII conformation. The holin protein of Mycobacteriophage D29 has two predicted transmembrane domains (1 and 2), of which only the first domain has been characterized thus far. $^{39,40}$ Here, we report our findings on the longest chame-leonic transmembrane domain 2 (TM2) sequence of 27 residues from D29 holin. We show that TM2 samples three secondary structures from a random coil to PPII-like conformation, and $\alpha$-helical structure, in an environment-dependent manner.

**MATERIALS AND METHODS**

**Materials**

Rink Amide AM resin (200–400 mesh, 0.63 mmol/g loading capacity) was purchased from Novabiochem (EMD Millipore Chemicals). 1-[Bis (dimethylamino) methylene]-1H-1,2,3-triazolo[4,5-b]pyridinium 3-oxid hexafluorophosphate (HATU) and Fmoc-labeled amino acids from GL Biochem (Shanghai). Anhydrous 1-hydroxybenzotriazole (HOBt) was purchased from SpectraChem Methanol (MeOH), ethanol (EtOH), isopropanol (iPrOH) and piperidine (PIP) were procured from Merck Specialities. Dimethylformamide (DMF), trifluoroacetic acid (TFA), N, N-diisopropylethylamine (DIPEA), thioanisole, 1,2-ethanedithiol (EDT), phenol, Kaiser test kit, N, N-dime-thyldodecylamine N-oxide (LDAO) were purchased from Sigma-Aldrich Co., LLC. All the lipids such as n-dodecylphosphocholine (DPC), 1,2-di-O-hexyl-sn-glycero-3-phosphocholine (DHPC), 1,2-didecanoyl-sn-glycero-3-phosphocholine (DDPC), 1,2-dilauroyl-sn-glycero-3-phosphocholine (DLPC), 1,2-dimyristoyl-sn-glycero-3-phosphocholine (DMPC), and 1,2-palmitoyl-sn-glycero-3-phosphocholine (DPPC) were procured from Avanti Polar Lipids. All other chemical and reagents were purchased from Merck Specialities and were of the highest purity available.

**Methods**

**Peptide Synthesis, Purification, and Mass Spectrometric Analysis.** We carried out the total chemical synthesis of the predicted 27-residue transmembrane domain 2 (TM2) from D29 holin, with the sequence H$_{2}$N-AAAANIDIVAGALNLVGAAPAATAVKCONH$_{2}$ (A33–K59). We followed Fmoc-based solid phase peptide synthesis, as reported earlier for the first transmembrane domain of holin, and used the Rink amide AM resin. $^{39}$ Activation of Fmoc-protected amino acids was done using HOBt, DIPEA, and HATU in dry DME. Fmoc removal was carried out using 20–30% piperidine after each successful coupling step. When the peptide chain length increased beyond 15 residues, the peptide synthesis reaction was shifted from 25 to 37°C, to improve amino acid coupling and Fmoc deprotection efficiency. After completion of synthesis, Fmoc was first deprotected. The peptide was cleaved from the resin and the side chains deprotected using the following cleavage cocktail containing free radical scavengers. 100 μl of the cocktail containing TFA: H$_{2}$O: phenol: thioanisole: EDT in the ratio of 82.5: 5: 5: 2.5 was used per mg resin, and the reaction was incubated for 8–10 h at 25°C. TFA and cleavage cocktail were filtered and evaporated on a rotary evaporator, and the peptide was precipitated using cold diethyl ether. The final air-dried peptide powder was resuspended in water and lyophilized multiple times to remove the residual cleavage cocktail reagents. The successful synthesis was confirmed by using mass spectrometric...
analysis, performed on a MALDI-ToF/ToF instrument (Bruker Daltonics). The spectrum is provided in Figure S1 (Supporting Information, SI).

**TM2 Refolding in Detergents, Lipids, and Bicelles.** Refolding of TM2 was carried out in a wide variety of in membrane mimetic environments, like micelles, lipids, and bicelles. We dissolved desired quantities of the peptide in TFE and used this solvent as a carrier to incorporate TM2 into membrane environments. The peptide + TFE mixture was added to desired amounts of the lipid or detergent dissolved in chloroform. Both solvents were co-evaporated by purging with N₂ gas, followed by a 12-h lyophilization. For refolding into micelles like LDAO (4–100 mM; critical micelle concentration (CMC) 1–2 mM), DPC (5–50 mM; CMC = 1.1 mM), and DHPC (100 mM; CMC = 15 mM), the lyophilized film was hydrated using the desired buffer, and the sample was heated, vortexed, and sonicated multiple times. For refolding into lipids (DDPC, DLPC, DMPC, and DPPC; all final lipid concentrations were maintained at 2.5 mM; CMC is in μM–mM), the buffer-hydrated film was first subjected to a 1-min cycle of heating at 45°C followed by vortexing, for 45 min. Then, the sample was subjected to freeze–thaw cycles using liquid N₂. Following this, the solution was extruded through a 100 μm membrane on a minietruder from Avanti Polar Lipids. The extrusion was repeated until the solution became translucent. A final sonication cycle was carried out at 45°C till the solution became clear (15 min for 10–14–C lipids and 30 min for the 16–C lipid). Refolding into bicelles (lipid-LDAO and lipid-DPC) was carried out vortexing the sample for 15–20 min, followed by 5–6 cycles of freeze–thaw using liquid N₂. Different bicelle q of 0.25, 0.5, 0.75, and 1.0 were obtained by maintaining a constant micelle concentration (4 mM for LDAO and 5 mM for DPC) and varying the lipid concentration according to the desired q.

After each refolding protocol, we pelleted down the particulate matter by centrifugation for 30 min at 18,000 × g at 10°C. TM2 does not have any aromatic residue that can be used for peptide quantification. We also faced interference of solvent or lipids, as well as problems in dissolving the peptide in aqueous solvents, for use in other quantification protocols. Hence, we used TM2 at concentrations corresponding to the highest solubility we could achieve in the respective solvents or buffers. In these preparations, we assumed that the peptide is largely monomeric in solution; however, we do not rule out the possibility that a population of TM2 peptides can exist as oligomers in solution.

**Circular Dichroism Measurements.** All circular dichroism (CD) experiments were carried out on a J-815 spectropolarimeter (JASCO, Japan) equipped with a six-cell sample chamber and a temperature-controlled Peltier set-up. CD spectra were recorded using a 0.1 cm path length quartz cuvette with a 100 nm/min scan rate, from 190 to 260 nm, with a 0.5 nm data pitch and a 1 s data integration time. Data, averaged over at least three accumulations, were blank subtracted and smoothed. Thermal melting scans were recorded between 5 and 95°C (5–65°C for samples with organic solvents) for samples prepared in aqueous media, at increments of every 5°C with a ramp rate of 1°C/min. Recovery from thermal denaturation was monitored from 95 to 5°C (or 65–5°C) for the same sample and wavelength scans were acquired using the parameters mentioned above. We could not accurately quantify the peptide concentration in each preparation, due to the absence of an intrinsic chromophore in the peptide. However, for any given sample, we maintained a similar dilution factor for its preparations. Data are reported directly as ellipticity values in units of mdeg.

**RESULTS AND DISCUSSION**

**Transmembrane Domain 2 Shows Conserved Ala- and Gly-Rich Sequence**

The predicted transmembrane domain 2 (TM2) of mycobacteriophage D29 holin spans residues 33–59 of the 141 residue protein. This region has a stretch of Ala repeats, comprising 11 of the total 27 residues of TM2; additionally, the sequence has three glycines (Figure 1A). Multiple sequence alignment with 12 other nonredundant mycobacteriophage holin proteins reveals that many of the Ala and Gly residues are conserved (Figure 1B). A highly conserved proline (residue 53) is also seen. Bioinformatics analysis of the predicted TM2 structure indicates that the sequence can adopt a helical structure at residues ~37–58. Predictions of the transmembrane region also point to a ~21-residue membrane spanning helix for TM2. Alanine possesses the highest propensity for helical φ-ψ values in soluble proteins; however, this helical propensity for Ala is lower in transmembrane peptides. On the other hand, Ala and Gly are two important residues that can adopt the PPII-like conformation in nonproline peptides. To examine the structural preferences of this Ala/Gly-rich transmembrane domain of holin, we synthesized the 27-residue TM2 peptide analog (A33–K59) using solid phase synthesis. We carried out a detailed spectroscopic characterization of synthetic TM2 using circular dichroism (CD) measurements in various solvents, detergents, and lipids to address the structural aspects of this peptide.
TM2 Shows Solvent-Dependent Structural Transition from α-Helix to PPII and Random Coil

Organic solvents are often used as mimetics of the membrane environment, and therefore, we first examined the structure of TM2 in MeOH, EtOH, iPrOH, and TFE. Far-UV CD measurements of TM2 in these solvents indicates that the peptide adopts α-helical conformation, with two negative maxima at 208 and 222 nm, and a positive maximum at ~195 nm (Figure 2A). The absolute ellipticity (θ in mdeg) varies linearly with peptide concentration, indicating that the concentration-adjusted CD spectrum (in molar ellipticity) is independent of the peptide concentration (Supporting Information Figure S2). We carried out a titration of the peptides from organic solvent to water and monitored the change in the far-UV CD profile. The spectra are summarized in Figure 2A. Here, MeOH → H2O represents the peptide stock prepared in MeOH and titration carried out with H2O. The lowest titration point (5% organic solvent) gives a CD spectrum with a negative maximum at 197 nm, which is considered as a characteristic PPII-like CD spectrum for non-Pro peptides. As the CD spectra of PPII-like structures and random coil (RC) are similar, we demarcate both structures in our study based on the ellipticity value at 197 nm and ~210 nm (Figure 3).

In the MeOH → H2O solvent titration, we see that TM2 undergoes a clear two-state transition from the helical structure in MeOH to a PPII-like CD profile in H2O, with an isodichroic point at 203 nm. We infer from the θ203 plot (ellipticity at 197 nm; Figure 2A, inset) that TM2 exhibits a

![FIGURE 2 Two-state conformational interconversion of TM2 in solvent titrations.](image-url)
conformational conversion between two defined structural elements, with a two-state profile. We observe the same phenomenon when we carry out solvent titration from EtOH-H₂O and iPrOH-H₂O, with isodichroic points at 203 and 204 nm, respectively. In the TFE-H₂O titration, the peptide retains a helical structure till ∼30% TFE, and is likely due to the stabilization of the α-helix by TFE. The helix–PPII transition is rapid, and is completed between 20 and 10% of TFE.

To assess the truly reversible nature of this structural transition seen in TM2, we carried out the reverse experiment, where we first dissolved the peptide in water. TM2 shows a CD spectrum corresponding to a RC when it is dissolved in water (Figure 2B). Here, H₂O → MeOH represents the peptide stock prepared in H₂O and titration carried out with MeOH. As the organic solvent concentration is increased in H₂O → MeOH, H₂O → EtOH and H₂O → iPrOH samples, we observe the structural transition from RC to α-helix, with some β-sheet content. At intermediate solvent concentrations, we observe a CD spectrum that resembles a β-sheet. When we compare the CD profiles for samples containing 95% organic solvent in Figures 2A and 2B, we notice that they are dissimilar. This data indicates that the conformation attained by TM2 depends on the initial solvation process. The transition from RC to α + β structure follows a two-state profile (Figure 2B, inset) and presents an isodichroic point at 205 nm. Interestingly, the H₂O → TFE titration shows a rapid conversion of the peptide from RC to α-helix, within a narrow concentration range. Hence, a TFE-induced helicity is observed for TM2, which is largely independent of the initial solvation condition of the peptide.

We carried out the thermal denaturation and recovery measurements for TM2 in organic solvents from 5 to 65–85°C (Supporting Information Figures S3, SI). We see no significant structural transition within this temperature range, and the marginal loss in secondary structure is recovered upon cooling. Hence, temperature does not influence the structural conversion seen in TM2. We conclude that TM2 undergoes a two-

Table 1  Summary of Predominant Secondary Structures Shown by TM2 Under Various Conditions

| Condition | Random Coil | β-Sheet | α-Helix | PPII |
|-----------|-------------|---------|---------|------|
| 100% organic solvent⁵ | | | | |
| (5% MeOH and TFE, 25% EtOH and iPrOH)⁶ | | | | |
| 100% H₂O | | | | |
| (95% H₂O or 5% organic solvent)⁷ | | | | |
| (5% H₂O or 95% organic solvent)⁷ | | | | |
| LDAO and DPC | | | | |
| SDS | | | | |
| 50 mM phosphate buffer pH 7.2 | | | | |
| DHPC | | | | |
| All lipids (DDPC, DLPC, DMPC, and DPPC) | | | | |
| Phosphocholine bicelles with LDAO and DPC | | | | |

⁵ All structures are deduced from the observed CD spectra at 25°C.
⁶ Here, organic solvent refers to MeOH, EtOH, iPrOH, or TFE.
⁷ These sample conditions were attained in the organic solvent → H₂O titration (e.g., MeOH → H₂O; Figure 2A).
⁸ These sample conditions were attained after H₂O → organic solvent titration (e.g., H₂O → MeOH; Figure 2B).
We examined the effect of detergent concentration on the far-UV CD spectra of TM2, using LDAO (4, 10, 20, 40, and 100 mM) and DPC (5, 10, 20, 30, and 50 mM). In LDAO samples (Figure 5, top left panel), we observe an increase in the overall ellipticity, which is accompanied by a red shift in the negative maximum from 200 to 204 nm, as the LDAO concentration increases. Similarly, in the case of DPC (Figure 5, top right panel) we observe an increase in the ellipticity (along with a marginally red-shifted negative maximum from 202 to 204 nm). The data suggests that increasing the detergent concentration (25-fold in LDAO and 10-fold in DPC) only causes a marginal increase in the local helical conformation. A large population of TM2 in solution, therefore, remains in the RC form (Table I). This is evident when we compare the CD spectra obtained for TM2 directly refolded in two concentrations of SDS (sodium dodecyl sulfate; 16 and 160 mM). TM2 displays the characteristic CD spectrum of a helix in SDS, due to the ability of SDS to induce a helical structure in transmembrane and membrane-associated proteins.

We monitored the change in secondary structure content of TM2 with temperature, in the various micelle concentrations (Supporting Information Figures S4, SI). We see a considerable loss in ellipticity at ~204 nm, whereas the corresponding data in organic solvents (Supporting Information Figures S3, SI) and SDS (Supporting Information Figures S5, SI) are not associated with ellipticity changes of similar magnitude. Hence, it

**FIGURE 5** Dependence of TM2 secondary structure on micelle concentration. Change in the secondary structure was monitored in 4–100 mM LDAO (top, left), 5–50 mM DPC (top, right) and 16–160 mM SDS (bottom). The lowest detergent concentration chosen is above the reported critical micelle concentration for each detergent. Low-to-high detergent concentration is represented as light-to-dark color in each spectrum. TM2 shows a local α-helical conformation with RC content in both LDAO and DPC. In SDS, a typical spectrum of a z-helix, with two negative maxima at 208 and 222 nm is seen.
is likely that micelles such as LDAO and DPC neither support the secondary structure of TM2 nor stabilize the scaffold.

**PPII-Like Structure Preserved in Lipids**

It is widely accepted that transmembrane proteins show superior structural characteristics in their native membranes or membrane mimetic environments. As TM2 is a predicted transmembrane domain, we folded the peptide into lipids, to examine the role of lipid chain length in promoting its native structure. In water, TM2 displays the RC conformation (Figure 6; also see Figure 2B), with a negative maximum at ~197 nm at 5°C. Upon thermal denaturation (till ~95°C), the CD spectrum shifts to a negative maximum at ~220 nm (Figure 6A, left panel). The change in the typical CD spectrum upon heating, which recovers on cooling the sample, suggests that TM2 forms soluble and reversible aggregates in water.

In phosphate buffer (pH 7.2), at low temperatures, we observe a PPII-like CD spectrum for TM2, with a negative maximum at 197 nm and a positive peak at ~216 nm (Figure 6A, middle panel). When we heat the sample, the PPII-like CD is lost, and we attain RC-like spectrum. Next, we examined the CD profiles of TM2 refolded in DLPC (left panel) and DMPC (right panel) buffered SUV preparations. In both (A) and (B), the spectra are represented as a gradient from light blue for low temperature (5°C) to dark blue for high temperature (95°C), at increments of 25°C. Inset ellipsities at 197 nm (200 nm in the case of DHPC) for melting (blue circles) and recovery (red circles) experiments. We obtain a RC conformation for TM2 in water, which shows the formation of soluble aggregates upon heating. In all other conditions, TM2 transitions from a PPII-like structure with a negative maximum at 197 nm, and a positive maximum at 216 nm. This result suggests that TM2 might remain as a soluble or membrane adsorbed peptide, and is less likely to be a transmembrane peptide.

**FIGURE 6** Understanding TM2 conformational changes in lipid vesicles. (A) Temperature-dependent change in TM2 conformation in water (left panel) and 50 mM phosphate buffer pH 7.2 (middle panel) and phosphate buffer containing DHPC micelles (right panel). (B) Temperature-dependent change in TM2 conformation when refolded in DLPC (left panel) and DMPC (right panel) buffered SUV preparations. In both (A) and (B), the spectra are represented as a gradient from light blue for low temperature (5°C) to dark blue for high temperature (95°C), at increments of 25°C. Insets show the ellipticity values at 197 nm (200 nm in the case of DHPC) for melting (blue circles) and recovery (red circles) experiments. We obtain a RC conformation for TM2 in water, which shows the formation of soluble aggregates upon heating. In all other conditions, TM2 transitions from a PPII-like structure with a negative maximum at 197 nm, and a positive maximum at 216 nm. This result suggests that TM2 might remain as a soluble or membrane adsorbed peptide, and is less likely to be a transmembrane peptide.

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denaturation and recovery measurements in all the conditions. The spectra in Figure 6 provide the representative CD wavelength scans for selected temperatures between 5 and 95°C (melting arm). Insets in Figure 6 summarize the melting (5–95°C) and recovery (95–5°C) data plotted at 197 nm. In all lipid and buffer conditions, TM2 adopts a residual helical structure at high temperatures up to 95°C (Table I). We observe the complete reversibility of the sample after thermal denaturation; additionally, the absence of hysteresis is evident from the overlapping data points in the melting and recovery experiments. Our observations are independent of the lipid used in the experiment. Hence, we conclude that TM2 adopts a PPII conformation in buffer, and retains this structure even in the presence of lipids. The absence of a clear lipid-dependent structural change allows us to conjecture that TM2 is likely to exist as a membrane-adsorbed species.

Mixed PPII and α-Helix Species Observed in Bicelles
In micelles, we observe a helical structure for TM2 (Figure 5), while in the presence of lipid vesicles, TM2 retains the PPII-like structure (Figure 6), and is likely to be retained in an SUV-adsorbed state. Hence, we examined the structural preference of TM2 when refolded in lipid bicelles. Unlike SUVs that exhibit curvature stress, bicelles and micelles present defects in the structure, which facilitates the insertion of guest protein molecules in the host lipidic system.

We refolded TM2 peptide into lipid bicelles prepared by doping detergents like LDAO and DPC in phosphocholines of increasing chain length. We also increased the bicelle size by varying the bicelle q (the ratio of long chain: short chain lipid) from 0.25 to 1.0 at 0.25 increments. Interestingly, we observe that TM2 adopts an intermediate structure (PPII + α-helix) in bicelles prepared using LDAO, and a more helical structure in bicelles prepared using DPC. Additionally, this structure is independent of the bicelle q and lipid chain-length (Table I, Figure 7 and Supporting Information Figures S7, S1).

We find that the structure of TM2 is largely influenced by the detergent used. The chain length and content of the long chain lipid (10-16–C) bears no influence on the TM2 structure. This data is in good agreement with our previous experiment in SUVs (Figure 6), wherein we also observe a chain length and temperature independent structural feature of TM2.

CONCLUSIONS
In the phage infection cycle, the last step involves the release of mature virions by disruption of the host cell membrane.47-49 The phage protein holin functions to perforate the host membrane and forms holes that are large enough to transport cell wall degrading enzymes from the host cytosol to the intermembrane space. It is known that the ‘hole formation’ is well-programmed to coincide with phage maturation, clearly suggesting that a trigger mechanism exists to activate inactive membrane-bound holin molecules to active pore-forming entities.49,50

In our previous study, we have observed that the first transmembrane domain of D29 mycobacteriophage holin can form holes in the membrane by means of a conformational switch.40 The role of the second transmembrane domain in facilitating pore formation is not yet known. It has recently been proposed that the C-terminal cytosolic domain of holin forms a coiled-coil motif that promotes holin-mediated bacterial cytotoxicity through oligomerization.51 Hence, it is likely that the second transmembrane domain facilitates the functioning of the other domains of holin: (i) conformational switch of the first transmembrane domain, and (ii) oligomerization of the C-terminal domain.

Through this study, we find that the second transmembrane domain of Mycobacteriophage D29 holin can adopt multiple conformations (α-helical and PPII-like forms) in solution. Recent structural studies of unfolded polypeptides have shown that the PPII conformation is a key feature for protein–protein

**FIGURE 7** Behavior of TM2 incorporated in lipid bicelles. Short chain (12-C) and long chain (14-C) lipids were mixed with 4 mM LDAO (panel A) or 5 mM DPC (panel B) in increasing amounts to obtain bicelles of varying q from 0.25 to 1.0. Shown here is the CD wavelength scans of TM2 incorporated in bicelles. In both lipidic bicelles, TM2 attains a predominantly RC conformation, with marginally greater helicity in DPC-containing bicelles (B). The spectra were not influenced by a change in the bicelle q, indicating that increase in lateral bilayer pressure does not alter TM2 structure.
interactions. Based on the proline- versus nonproline PPII conformations, CD spectra with different positive and negative maxima are observed, and both structures are widely accepted. The conserved Ala- and Gly-rich sequence of TM2 suggests that these residues are essential to dictate the formation of a Pro–PPII-like form in this peptide, under favorable conditions.

Environmental factors such as solvent conditions influence the formation of \( \alpha \)-helical or PPII-like structures. For example, we observe that TM2 adopts a helical structure when dissolved in water or organic solvents and detergent micelles. TM2 transitions to a PPII-like structure in the presence of double-chain lipid micelles and SUVs.

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Figure 8 Environment-dependent modulation of TM2 structure. (A) Schematic representation of the overall shape of DPC and DHPC molecules. (B) The observed structures of TM2 range from \( \alpha \)-helix in the presence of organic solvents and detergent micelles. TM2 transitions to a PPII-like structure in the presence of double-chain lipid micelles and SUVs.

Double-chain phospholipids (DHPC, 10-16–C phosphocholines) also present a hydrophobic environment; however, TM2 is unable to incorporate in these vesicles. It is worth mentioning here that DHPC can also form micellar structures. However, DHPC micelles are different from detergents, as the latter possess a single hydrocarbon chain. The overall structures of the two molecules in solution are therefore different—a single DHPC molecule adopts a cylindrical structure while a single LDAO or DPC molecule adopts an inverted cone-shaped form (Figure 8A). A cylindrical form is also seen for the long chain phosphocholine lipids. This difference influences the TM2–lipid packing efficiency, as a result of which we observe a detergent- or organic solvent-coated TM2 helix, while TM2 remains in a membrane adsorbed PPII-like state when presented with double-chain phosphocholines.

What are the implications of the structural transitions of TM2 due to solvation and hydration? The two-state model observed in our solvent titration experiments can be compared with the Zimm–Bragg and Lifson–Roig helix–coil theory proposed to explain the phenomenon of structural transition between unfolded and helix states (the unfolded state that we considered here is PPII or RC). When the first transmembrane domain is inserted into the membrane, the membrane defect is likely to allow the insertion of TM2—hence, the membrane localization of TM2 will be assisted by the first transmembrane domain. TM2 does not possess the conserved GX2/3G motif required for helix dimerization, nor does it show any propensity for a coiled-coil motif. Hence, it is unclear whether TM2 can form homo- or hetero-oligomers in vivo. We do find that TM2 has a short hydrophobic segment (Supporting Information Figure S8), which might facilitate TM2 localization in the membrane. In this hydrophobic environment, TM2 will adopt a helical structure (Figure 8B), similar to what we obtain in organic solvents or detergent micelles (see Figures 2 and 5). When pore formation occurs, it is postulated that TM2 can exit the membrane. In this state, we believe that TM2 will be membrane-adsorbed and adopt a PPII-like structure (Figure 8B), similar to our results from SUVs (Figure 6).

Functional studies show that both full-length holin and TM2-less holin proteins show a similar effect on cell toxicity. Clearly, TM2 is not involved in the toxicity. Preliminary electrophysiology measurements of TM2 in planar lipid bilayers also suggest that this peptide cannot incorporate itself in lipid membranes (unpublished observations). Therefore, we speculate that TM2 co-localizes with the first transmembrane domain when holin is transported to the host inner membrane. When the trigger for pore formation occurs, TM2 is likely to exit the membrane, change conformation, and strengthen the transmembrane pore assembly by promoting the oligomerization of the C-terminal domain. This structural transition TM2 may promote the holin mediated pore formation in the cytoplasmic membrane of bacteria. Further studies in this direction are now required to validate the role of TM2 in holin function.

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