Characterizing the structure–function relationship reveals the mode of action of a novel antimicrobial peptide, P1, from jumper ant Myrmecia pilosula†

Tien-Sheng Tseng,a Keng-Chang Tsai,b,c and Chinpan Chen†a,b,c

Microbial infections of antibiotic-resistant strains cause serious diseases and have a significant impact on public health worldwide, so novel antimicrobial drugs are urgently needed. Insect venoms, a rich source of bioactive components containing antimicrobial peptides (AMPs), are attractive candidates for new therapeutic agents against microbes. Recently, a novel peptide, P1, identified from the venom of the Australian jumper ant Myrmecia pilosula, showed potent antimicrobial activities against both Gram-negative and Gram-positive bacteria, but its structure–function relationship is unknown. Here, we used biochemical and biophysical techniques coupled with computational simulations to explore the mode of action of P1 interaction with dodecylphosphocholine (DPC) micelles as a model membrane system. Our circular dichroism (CD) and NMR studies revealed an amphipathic α-helical structure for P1 upon interaction with DPC micelles. A paramagnetic relaxation enhancement approach revealed that P1 orients its α-helix segment (F6–G14) into DPC micelles. In addition, the α-helix segment could be essential for membrane permeabilization and antimicrobial activity. Moreover, the arginine residues R8, R11, and R15 significantly contribute to helix formation and membrane-binding affinity. The lysine residue K19 of the C-terminus functionally guides P1 to interact with DPC micelles in the early interaction stage. Our study provides insights into the mode of action of P1, which is valuable in modifying and developing potent AMPs as antibiotic drugs.

1. Introduction

The rapid emergence of resistant bacterial strains pushes classical antibiotics under intense pressure and causes severe public health problems worldwide.1,2 Thus, novel biooidal agents without the likelihood of evolving resistance are urgently needed. Antimicrobial peptides (AMPs), evolutionarily ancient components of host innate defense against harmful microorganisms, are widespread in bacteria, protozoa, plants, and animals.3–5 They function to kill a broad spectrum of microorganisms including multi-drug-resistant (MDR) bacteria by targeting and disrupting the plasma membrane.6–8 Given the progressive increase in MDR pathogens, AMPs and their derivatives are thought to have substantial potential as therapeutic agents.

To date, more than 2000 distinct AMPs have been characterized and deposited in the Antimicrobial Peptide Database (AMSDb, http://aps.unmc.edu/AP/main.php). This database also serves as a platform to predict the structure, function, and antimicrobial activity of any queried sequences.4,9,10 In general, AMPs are small amphipathic cationic peptides (12–30 amino acids) consisting of positive charges from +2 to +9 as well as some hydrophobic residues.7,11,12 In nature, AMPs exist in four main structural types: loop, α-helical, β-sheet, and extended peptides.12,13 The α-helical conformation is the most abundant and successful structural arrangement observed at the microbial surface in innate defense.13 Functionally, almost all AMPs share two important characteristics: (1) the ability to assume an amphipathic helical structure (most AMPs are linearly unstructured in solution and when binding to the target membrane, they fold into an amphipathic helix) and (2) a net cationicity (AMPs exert their cell lytic ability by first binding to the negative-charged microbial surface).12,13–19 With these functionally important properties, AMPs can break down the transmembrane potential, disrupt the balance of ion gradients, and cause the leakage of cell contents, ultimately leading to microbial death.

In nature, venom of arthropods is rich in bioactive compounds.20 Particularly, ant venom is a great source of antimicrobial molecules,
functioning to prevent infection by microorganisms in the colon.\textsuperscript{20,21} Among these molecules, AMPs have been found in several species, such as ponericin from the neotropical ant \textit{Pachycondyla goingii}\textsuperscript{21} and pilosulin from the Australian \textit{Myrmecia pilosula}.\textsuperscript{22–24} These AMPs display potent antimicrobial activities against both Gram-negative and Gram-positive bacteria. Since the discovery of AMPs from ant venom, their biological activities have been suggested for potential use in therapeutic applications.\textsuperscript{25,26} Recently, Igor et al. identified a new potential antimicrobial peptide segment, P1, from pilosulin-1 of the jumper ant \textit{M. pilosula}.\textsuperscript{27} P1 has broad-spectrum antimicrobial activity, including against standard and MDR bacteria. Although P1 was found in an \(\alpha\)-helical conformation in the trifluoroethanol (TFE)-mimetic hydrophobic environment, detailed structural characterization and the structure–function relationship are unclear. The development of AMPs as biocidal agents is limited and scientifically a challenge due to lack of detailed information on their physicochemical properties, which are highly associated with their mechanism of action.\textsuperscript{28,29} Therefore, to unveil the mode of action of P1, we need to investigate its structural properties, ability to interact with membranes, and solution structure on binding with membrane-mimetic micelles.

In this study, we used biochemical and biophysical methods coupled with molecular computation to disclose the physicochemical properties of P1 upon interacting with dodecylphosphocholine (DPC) micelles. Such methods included far-UV CD, dye-leakage fluorescence assay, 2D and spin-labeling NMR, and molecular dynamics (MD) simulations to estimate the secondary structure content, membrane-permeabilizing activity, solution structure, orientation in DPC micelles, and peptide–micelle model of P1. These observed structural and functional insights explain the mode of action of P1 against microbes and provide valuable information for the development of new anti-infective agents.

2. Materials and methods

2.1. Materials

Peptide P1 (GLGSVFGLARRGLGRVIPKV) was synthesized by Kelowna International Scientific, Taipei. 1-Palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC) and 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoglycerol (POPG) were purchased from Avanti Polar Lipids (Alabaster, AL, USA). Calcein, MnCl\(_2\), and 5-, 12- and 16-doxyl stearic acids were obtained from Sigma-Aldrich. DPC-d\(_{10}\), methanol-d\(_4\), and D\(_2\)O were obtained from Cambridge Isotope Laboratories. Sodium dodecyl sulfate was purchased from Merck (Darmstadt, Germany).

2.2. Methods

2.2.1. Circular dichroism (CD) spectroscopy. Far-UV CD experiments involved the use of an Aviv CD 202 spectrometer (Lakewood, NJ). P1 was liquified in 10 mM sodium phosphate buffer (pH 5.0) to yield a 1.5 mM stock. Ratios for CD samples were, for P1 (60 \(\mu\)M) in 20% and 40% TFE, P1: DPC = 1:100 (molar ratio) and P1: SDS = 1:100 (molar ratio) prepared by diluting the P1 peptide stock (1.5 mM) into TFE, DPC, and SDS separately to reach the final concentrations and molar ratios of interest. Far-UV CD spectra were recorded by using a 1 mm path length quartz cuvette at 25 °C with the observed wavelength range between 190 and 260 nm. All spectra were averaged over three scans and converted to the mean residue ellipticity, \([\theta]\).

2.2.2. Preparation of large unilamellar vesicles (LUVs). The extrusion method reported by Wei et al.\textsuperscript{30} was used to prepare LUVs. The phospholipids were dissolved in chloroform and completely dried in a nitrogen atmosphere. The prepared dried lipid film was dissolved in phosphate-buffered saline (PBS) (137 mM NaCl, 2.7 mM KCl, 10 mM Na\(_2\)HPO\(_4\), 1.8 mM KH\(_2\)PO\(_4\), at pH 7.4) and subjected to 10 freeze–thaw cycles. To generate the LUVs, the lipid suspensions were extruded 10 times by using a mini-extrusion device (Avanti Polar Lipids, Alabaster, AL, USA) through two stacked 0.4 \(\mu\)m pore-size polycarbonate filters, and then extruded another 10 times using two stacked 0.1 \(\mu\)m pore-size filters. Similarly, calcein-entrapped LUVs were generated in calcein-containing buffer (70 mM calcein, 10 mM Tris, pH 7.4) by the same process mentioned above. Moreover, the unentrapped calcein was eliminated by centrifugation (10,000 rpm for 10 min) three times by using isosmotic buffer (10 mM Tris and 100 mM NaCl, pH 7.4). Finally, the size of the generated LUVs was confirmed by dynamic light scattering on a Zetasizer Nano ZS (Malvern Instruments, Malvern, UK).

2.2.3. Calcein leakage assay. Peptide-induced calcein leakage, as reflected by an increase in fluorescence, was examined using a JASCO FP-8500 spectrofluorometer (JASCO, Tokyo) at excitation and emission wavelengths of 496 and 515 nm. Measurements involved the use of \(30 \mu\)M lipids of the calcein-entrapped LUVs in 20 mM Tris and 100 mM NaCl, pH 7.4, at 25 °C. We induced 100% leakage in 3 min by the addition of 0.1% (v/v) Triton X-100. The degree of leakage induced by various concentrations of peptides was calculated with the following equation: % leakage = \([F - F_0]/(F_t - F_0)\) × 100, where \(F_0\) and \(F_t\) are the initial fluorescence intensities observed without the peptide and after Triton X-100 treatment, respectively.

2.2.4. NMR spectroscopy. The NMR samples were prepared by mixing P1 (final concentration 1.5 mM) with DPC (final concentration 150 mM) to reach a molar ratio of 1:100, consisting of 10 mM sodium phosphate and 10% D\(_2\)O at pH 5.0. The pH values of the samples were adjusted before NMR measurements. All NMR experiments were performed at 310 K using 600 and 800 MHz Bruker Avance spectrometers. 2D-NOESY spectra were acquired at two distinct mixing times of 150 and 300 ms. TOCSY spectra were recorded with a mixing time of 60 ms at 2048 points in \(t_2\) and 320 points in \(t_1\). A natural abundance \(\left(^{1}H,^{15}N\right)\) HSQC spectrum\textsuperscript{31} was collected at 2048 points in \(t_2\) and 400 points in \(t_1\). The spectra were processed by using TopSpin 3.1 (Bruker Spectrospin), NMRPipe\textsuperscript{32} and Sparky (T. D. Goddard and D. G. Kneller, University of California, San Francisco).

2.2.5. Structure calculation. 2D-NOESY spectra (150 ms mixing time) for P1 in DPC micelles (molar ratio 1:100) were recorded at pH 5.0 and 310 K to obtain distance constraints by manually assigning NOE cross-peaks. On the basis of the
TOCSY spectra were acquired at 310 K with 2048 data points in and 15N chemical shifts from the natural abundance HSQC recording the $^1$H–$^1$H TOCSY spectra. For the PRE effect of P1–micelle solution and equilibrated for 15 min before were added to reach final concentrations of 0.5 and 1 mM in was placed in a 54 Å$^3$ periodic box of spc216 water. A total of 50 placed at the DPC–water interface. The peptide–micelle system was placed in the Tieleman laboratory (http://moose.bio.ucalgary.ca). Our atom parameter set, lipid.itp, and the pre-equilibrated micelles were used for the peptide and solvent. The GROMOS united-force field parameters were calculated on the basis of established protocols. All parameters were the same except for probe tuning and field shimming. The cross-peak intensities of probe tuning and field shimming. The cross-peak intensities of peptide were measured with and without paramagnetic solutions and calculated on the basis of established protocols. MD simulations. MD simulations involved the use of GROMACS v4.6.7. The GROMOSE96 force field parameters were used for the peptide and solvent. The GROMOS united-atom parameter set, lipid.itp, and the pre-equilibrated micelles (65 DPC molecules) with 6305 water molecules were downloaded from the Tieleman laboratory (http://moose.bio.ucalgary.ca). Our determined solution structure of P1 was used as the starting conformation for the simulation. The P1 peptide was initially placed at the DPC–water interface. The peptide–micelle system was placed in a 54 Å$^3$ periodic box of spc216 water. A total of 50 chloride and 46 sodium counter ions were added as 150 mM electrolytes. The P1 peptide was positionally constrained and subjected to conjugated gradient minimization to eliminate interfering contacts. The simulation was thermostated at 310 K and the temperature was coupled with the Berendsen algorithm (coupling constant, 0.1 ps). Electrostatic interactions and the cut-off values for van der Waals interactions were calculated with the particle-mesh Ewald algorithm. The steepest decent algorithm was used for energy minimization down to a maximum gradient of 1000 kJ mol$^{-1}$ nm$^{-1}$. NVT-ensemble state conduction was used for equilibrium, in which heavy atoms of peptide were positionally restrained by applying a spring constant of 1000 kJ mol$^{-1}$ nm$^{-2}$. Additionally, all bonds were constrained with the LINCS algorithm. After that, isothermal-isobaric (NPT) equilibration was performed for 10 000 ps with the Parrinello–Rahman algorithm (coupling constant, 0.5 ps). The position restraint used in NPT was also used in NPT equilibration. The MD simulation was performed for 130 ns with an NPT ensemble.

3. Results

3.1. CD spectroscopy

Far-UV CD spectroscopy was used to estimate the secondary structure of P1. The CD spectra of P1 appeared as a random coil conformation in buffer solution (10 mM sodium phosphate, pH 5.0). However, P1 showed an $\alpha$-helical conformation in 20% TFE ($\alpha$-helical content 28.2%) (Fig. 1). The CD spectra of P1 in 40% TFE showed strong signals at 208 and 222 nm ($\alpha$-helical content 63.5%). When interacting with DPC micelles (peptide: DPC molar ratio 1:100), P1 was less helical ($\alpha$-helical content 58.5%) than with 40% TFE. When interacting with SDS micelles (peptide: SDS molar ratio 1:100), P1 showed a slightly lower $\alpha$-helical content (57.6%) than with DPC micelles. Notably, P1 also displays helical conformations in neutral and anionic liposomes, with $\alpha$-helical contents 42.1 and 71.3% to POPC and POPG LUVs, respectively (peptide: liposome molar ratio 1:20), as shown in Fig. 2A.

3.2. Solution structure of P1 in complex with DPC micelle

NMR experiments were performed to determine the solution structure of P1. P1 showed poorly dispersed and low-resolution spectra in negative-charged SDS micelles (data not shown). However, the cross peaks from both 2D-NOESY and TOCSY were well dispersed in DPC micelles at pH 5.0 and 310 K.
The solution structure of P1 was determined in DPC micelles. The sequential assignments involved 2D-TOCSY and NOESY spectra. The 239 NOE-derived distance constraints consist of 82 intraresidues, 86 sequentials, and 71 medium-range distance restraints. In addition, 32 backbone dihedral angles derived from $^1$H, $^{15}$N-HSQC spectra (Fig. S1, ESI) were used for the structure calculation of the micelle-bound P1 (Table 1). An overlapped ensemble of the 20 lowest-energy structures is presented in Fig. 3C. The root mean square deviation calculated from the averaged coordinate was 0.40 ± 0.13 Å for backbone heavy atoms and 0.84 ± 0.19 Å for all heavy atoms (Table 1). Furthermore, a Ramachandran plot produced using...
### Table 1 NMR structure calculation parameters (PDB ID: 5X3L)

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<tr>
<th>Type of Restraint</th>
<th>Number</th>
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<td>239</td>
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<tr>
<td>Dihedral angle restraints</td>
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**Ramachandran plot summary (%)**
- Most favored: 83.2%
- Additionally allowed: 16.8%
- Generally allowed: 0%
- Disallowed: 0%
- Average RMSD from the mean structure:
  - Back atoms: 0.40 ± 0.13 Å
  - All heavy atoms: 0.84 ± 0.19 Å

The default parameters and force constants of protein-allhdg.param and annel.inp were used for the structure calculations. There are no NOE and dihedral angle violations observed during the structure calculations.

PROCHECK demonstrated that 83.2% and 16.8% of P1 residues were located in the most favored and additionally allowed regions, respectively. Table 1 shows the detailed structural statistics for P1. The overall structure of P1 consists of an apparent three-turn α-helix (residues S4−R15) with an extended-loop conformation observed in both N- and C-termini (Fig. 3D). The structure of P1 is clearly amphipathic—the positive-charged side chains of arginine and lysine residues (R8, R11, R15, and K19) are on one side of the helix and the hydrophobic side chains of the remaining residues are on the other side (Fig. 3D). The NOE connectivity diagram (Fig. S2, ES†) consistently indicated an α-helical structure covering residues S4−R15 as well. Such an amphipathic helix, composed of positive-charged side chains (Fig. 3D and E), attracts negative-charged bacterial membranes.

### 3.4. Localization of P1 in DPC micelles

We further used PRE experiments to probe the localization of P1 in DPC micelles. The spin-labeled fatty acids 5-, 12-, and 16-DSAs, with the paramagnetic doxyl-group at the 5th, 12th, and 16th carbon positions of the acyl chain, respectively, were used. 5-DSA affects the NMR signals of NH-C6H that are near the surface of the micelle; 12- and 16-DSAs perturb the NMR signals of NH-C6H that are inserted or buried into the micelle. In PRE experiments, the cross peaks of NH-C6H resonances (I2 dimension) in TOCSY were used to analyze the relative intensity decrease compared to that of the peptide in DPC micelles without spin label effects. The result showed that the mean remaining amplitudes of P1 in the presence of 5-, 12-, and 16-DSAs were 22, 14, and 20%, respectively (Fig. 4A). Residues V5−G14 were significantly interfered by 5-, 12-, and 16-DSAs (90% of the remaining amplitudes were smaller than the average), so this segment is probably inserted into the DPC micelles. However, the remaining amplitudes of the N- (L2−S4) and C-termini (R15−V20) were mostly higher than the average, indicating that the termini are outside DPC micelles and exposed to the solvent.

### 3.5. MD simulation of P1 in DPC micelles

The PRE results revealed the possible localization of P1 in the DPC micelles; however, a detailed interaction between P1 and DPC micelles is not evident. Therefore, we performed MD simulation to monitor the atomic interactions between P1 and DPC micelles. Initially, the determined P1 solution structure was positioned at the solvent–hydrophobic interface of the DPC micelle at the beginning of the simulation. The conformational change of P1 upon interaction with the DPC micelle was monitored by calculating the RMSD of backbone atoms during the simulation (Fig. 5B). During the 130 ns simulation, the backbone RMSD fluctuated between 1.5 and 4.7 Å. The entire picture of P1 interacting with DPC micelles as a function of simulation time is shown in Fig. 5A. The structure of the P1−micelle complex obtained at 25 ns showed a less compact α-helical conformation. In this conformation, the amphipathic P1 bound to the surface of the DPC micelles mainly by the hydrophobic side. Meanwhile, the entire structure was tethered with hydrogen bonds contributed by residues G3, S4, R11, R15 and K19, which stabilize the interaction between P1 and the DPC micelle. At 50 ns, the C-terminus of P1 gradually merged into the micelle. At 75 ns, the C-terminus kept moving deep into the micelle, and the binding of the whole peptide was enhanced by the positive side chains of arginine residues electrostatically interacting with the heads of phospholipids. At 100 ns, more than 50% of the P1 structure was buried in the micelle, except that the K19, R8, and R11 residues remained exposed to the solvent. At 130 ns, P1 bound to an apparently distorted DPC micelle, with the buried middle α-helical segment (F6−G14) and a small amount of solvent exposing the N- and C-termini. Furthermore, the interactions between P1 and the DPC micelle were quantified by monitoring the distance between the center of mass of the peptide backbone and the center of mass of the micelle acyl chains as a function of simulation time (Fig. 5C).

### 4. Discussion

Ants are social insects with elaborate defensive systems to prevent infection by microorganisms. In some ant species, the venom gland secretions containing AMPs play a vital role in...
Fig. 4  The position of P1 in DPC micelles. (A) The remaining amplitudes of NH-CαH cross peaks derived from the TOCSY spectra of P1 due to 5-, 12-, and 16-DSAs. The concentration of each DSA corresponds to a spin label per micelle. The cyan, blue, and gray dotted lines indicate the mean remaining amplitudes of P1 in the presence of 5-, 12-, and 16-DSAs, individually. (B) The positions of the N- and C termini and the middle segment of P1 versus molecular dynamics simulation time quantified by distance from the center of mass of Cα atoms to the center of mass of the micelle acyl chain. The red, green, and black lines denote the distance from the center of mass of Cα atoms of the N-terminus (G1–S4), C-terminus (R15–V20), and middle segment (V5–G14) to the center of mass of the micelle acyl chain, respectively.

Fig. 5  MD simulation with P1 in DPC micelles. (A) Image of P1 interacting with DPC micelle presented with snapshots at 0, 25, 50, 75, 100, and 130 ns simulation times. The DPC lipid is presented as spheres in white with the phosphorus and oxygen atoms in black and gray, respectively. P1 is shown as a cyan ribbon with arginine and lysine residues in blue. (B) P1 structure versus simulation time quantified by backbone RMSD to the NMR structure. (C) P1 position versus simulation time quantified by distance from the peptide backbone center of mass to the micelle acyl chain center of mass.
The sting of an endemic Australia ant, *M. pilosula*, frequently causes insect allergy and death by envenomation. In addition to various enzymes and pharmacologically active components, the venom of *M. pilosula* contains peptides named pilosulins. P1, the N-terminal 20 residues of pilosulin-1, shows potent and broad-spectrum antimicrobial activity, but its mode of action against microbes was unclear. Here, we investigated the mode of action of P1 interaction with DPC micelles by using biochemical, biophysical, and computational methods. Our CD and NMR studies revealed an amphipathic \( \alpha \)-helical structure for P1 when binding to DPC micelles. Our PRE approach revealed that P1 orients its \( \alpha \)-helix segment (F6–G14) into DPC micelles. Besides, the \( \alpha \)-helix segment could be essential for membrane permeabilization and antimicrobial activity. Moreover, the R8, R11, and R15 residues significantly contribute to helix formation and membrane-binding affinity and the K19 residue could functionally guide P1 to interact with DPC micelles in the early binding stage. Our study provides insights into the mode of action of P1, which is helpful in developing and modifying AMPs as new and potent antibiotic drugs.

We firstly characterized the structural property of P1 associated with its bactericidal function by CD spectroscopy. P1 presents a random coiled feature in aqueous solution but an \( \alpha \)-helical conformation in a 40% TFE–water solvent system. Additionally, P1 has characteristic \( \alpha \)-helical structures in negative-charged SDS, zwitterionic DPC micelle systems (Fig. 1), POPC (neutral) and POPG (highly negative-charged) LUVs (Fig. 2A), revealing an induced disordered-to-ordered conformational transition upon binding to phospholipid membranes. These observations agree with previous reports that under aqueous conditions, AMPs are disordered, and when bonded to phospholipid membranes, they become structured. Thus, P1 is bioactive in the \( \alpha \)-helical conformation to interact with bacterial membranes. It is noteworthy that the helical conformations of P1 adapted in the POPC and POPG LUVs differ both qualitatively and quantitatively. The \( \alpha \)-helical contents of P1 in the POPC and POPG LUVs increased from 14.6 to 42.1% and 37.7 to 71.3% as the peptide:liposome molar ratio increased from 1:10 to 1:20, respectively (Fig. 2A). Obviously, the anionic liposomes induced more helicity to P1 than the neutral ones and it appeared that the helicity was induced in a liposome concentration-dependent manner. Suggestively, this may be caused by aggregations or oligomerizations as the amount of peptide in the membrane increased, pointing at the importance of electrostatic interactions of P1 toward anionic membranes. To disclose the structural basis for the P1 mode of action upon interaction with the microbial membrane, we determined its solution structure by \( ^1 \)H NMR in the presence of DPC micelles. The CD result aligned with the well-dispersed 2D-NOESY and TOCSY spectra (Fig. 3) demonstrates that P1 has a well-defined \( \alpha \)-helix conformation upon interaction with DPC micelles. Structurally, residues S4–R15 of P1 assume an amphipathic \( \alpha \)-helix with positively charged and hydrophobic side chains located on opposite sides. Functionally, P1 exerts strong membrane-disruptive activity against negatively charged phospholipid vesicles as seen from our dye leakage experiments (Fig. 2B). P1 induced 100% calcein leakage at peptide concentrations of 0.15 and >0.6 \( \mu \)M in turn for the POPG and POPC LUVs. This result elucidates the selective activity of P1 against negatively charged bacteria membranes rather than mammalian cells, consistent with the observed antimicrobial (MIC = 2–8 \( \mu \)M) and hemolytic activities (10–100 \( \mu \)M causing 20–100% lysis). Notably, the POPG LUVs induced more helix formation of P1 than the POPC LUVs (Fig. 2A) as seen from our CD approach supporting the observed selective membrane-permeabilizing ability of P1. As reported by Chou et al., the \( \alpha \)-helicity of AMPs is significantly associated with their dye-leakage capabilities and antibacterial activities. Accordingly, the induced \( \alpha \)-helices upon binding with phospholipid membranes, such as S4–R15 of P1 in DPC micelles, could play a critical role in its membrane permeabilization and bactericidal activity.

Generally, the amphipathic helix of AMPs more likely binds to the interfacial region of bacterial membranes with the polar region exposed to the solvent and the hydrophobic face embedded in the membrane. Our PRE results (Fig. 4A) demonstrated that the \( \alpha \)-helical segment (F6–G14) of P1 is buried, possibly with the hydrophobic residues oriented toward the micelle interior and the positively charged residues (R8, R11, and R15) facing towards the surface of the micelle. However, the localizations of both N- (L2–G3) and C-termini (R15–V20) may not be well demonstrated by the PRE results. Therefore, we performed the MD simulation to monitor the interactions and positions of P1 in DPC micelles. Our MD simulation revealed that at 25 ns, P1 was attached and anchored onto the surface of DPC micelles with mainly hydrophobic contacts and electrostatic interactions of R11, R15 and K19. During 50–75 ns, the K19 residue guided the C-terminus of P1 to firstly bury into the micelle, further with the aid of R8, R11, and R15 to electrostatically stabilize the entire binding. These observations indicate that the \( \alpha \)-helix segment (F6–G14) and the charged residues (R8, R11, R15, and K19) could be of great importance in binding and permeabilizing the bacterial membranes. In addition, the localization of the N-terminus of P1 is farther from the center of the DPC micelle (the average distance is \( \geq 2.5 \) nm) during the simulation time compared to that of the middle segment (the average distance is \( \leq 1.5 \) nm) (Fig. 4B). This demonstrates that the N-terminus is most likely exposed to the solvent. The position of the C-terminus fluctuates considerably. It is far from the center of the micelle between 0 and 60 ns, and comes closer during 70–100 ns, finally reaches a distance of about 2 nm at 130 ns. This observation indicates that the C-terminus of P1 could be outside the micelle. The only observed difference is that the well-defined \( \alpha \)-helix of P1 is shorter in the MD simulation (F6–G14) than in the NMR determined (S4–R15). This deviation is reasonable because both NMR and MD simulation have their individual limitations. The NMR can reveal the real experimental structure of P1 with reliable precision, while the MD simulation can capture the hidden discrete conformations and behaviors of P1 interacting with DPC micelles. Thus, we propose that the \( \alpha \)-helix (F6–G14) of P1 could be functionally essential and bioactive in disrupting and permeabilizing bacterial membranes for antimicrobial activity.

P2, a variant of P1, shows enhanced antibacterial and reduced hemolytic activity. In sequence, the P1 amino acids G3, V5, I12,
and G14 are mutated to the P2 amino acids L3, K5 K12, and L14, respectively. With the additional lysine residues (K5 and K12), P2 (charge, +7) is more positively charged than P1 (charge, +5). Although the increased cationicity of P2 may promote its interaction with the negative-charged microbial surface, the extra positive charges disrupted the original amphipathic nature of the helix (Fig. S3, ESI†). This change also greatly altered the mean hydrophobicity of P1 (from 0.68 to −0.28) and reduced the helicity of P1 when exposed to 10 mM SDS, for only minor improvement in antibacterial activity.27 The conserved R8, R11, and R15 residues are structurally located at the z-helical segment of P1 and functionally involved in an electrostatic interaction with DPC micelles observed in our MD simulation. Therefore, these arginine residues are essential for the helical formation and significantly contribute to the membrane-binding ability of P1.

5. Conclusion

In this study, we explored the mode of action of M. pilosula P1 against microbes by biochemical and biophysical techniques. P1 undergoes a disordered-to-ordered transition when binding to lipid membranes such as DPC micelles and maintains a well-defined z-helical structure in DPC micelles. We have determined the first solution structure of P1 in complex with DPC micelles. P1 orients itself into DPC micelles, with its N- and C-termini exposed to the solvent, and the central z-helix segment (F6–G14) buried into the micelles. P1 exerts its selectivity against negative-charged bacterial membranes; especially, its z-helix segment (F6–G14) could be critical for membrane permeabilization and antimicrobial activity. The conserved arginine residues R8, R11, and R15 are highly associated with helical conformation formation and membrane-binding affinity. The C-terminus lysine residue K19 could be functionally important in guiding P1 to merge into the DPC micelle in the early binding stage. Collectively, our study gives insights into the mechanism of action and functionally important residue annotations of P1, which may be of great use in developing new therapeutic agents against microbes.

Conflicts of interest

The authors declare no competing financial interests.

Acknowledgements

We thank the National Center for High-Performance Computing, Taiwan, for computer time and facilities. The Discovery Studio computations were performed at the National Center for High-Performance Computing, Taiwan. We acknowledge the use of the Chirascan-Plus CD Spectrometer in the Biophysics Core Facility, Department of Academic Affairs and Instrument Service at Academia Sinica. We also acknowledge the acquisition of NMR spectra at the High-field Biomacromolecular NMR Core Facility, Academia Sinica. This work was supported by Academia Sinica [104-0210-01-09-02] and the Ministry of Science and Technology, Taiwan, ROC [MOST 103-2311-B-001-026-MY3 and MOST 105-2320-B-001-019-MY3]. We thank Laura Smoakers for copyediting the manuscript.

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