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Structure and orientation of antibiotic peptide alamethicin in phospholipid bilayers as revealed by chemical shift oscillation analysis of solid state nuclear magnetic resonance and molecular dynamics simulation

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ABSTRACT

The structure, topology and orientation of membrane-bound antibiotic alamethicin were studied using solid state nuclear magnetic resonance (NMR) spectroscopy. ^{13}C chemical shift interaction was observed in [1-^{13}C]-labeled alamethicin. The isotropic chemical shift values indicated that alamethicin forms a helical structure in the entire region. The chemical shift anisotropy of the carbonyl carbon of isotopically labeled alamethicin was also analyzed with the assumption that alamethicin molecules rotate rapidly about the bilayer normal of the phospholipid bilayers. It is considered that the adjacent peptide planes form an angle of 100° or 120° when it forms α-helix or 3_{10}-helix, respectively. These properties lead to an oscillation of the chemical shift anisotropy with respect to the phase angle of the peptide plane. Anisotropic data were acquired for the 4 and 7 sites of the N- and C-termini, respectively. The results indicated that the helical axes for the N- and C-termini were tilted 17° and 32° to the bilayer normal, respectively. The chemical shift oscillation curves indicate that the N- and C-termini form the α-helix and 3_{10}-helix, respectively. The C-terminal 3_{10}-helix of alamethicin in the bilayer was experimentally observed and the unique bending structure of alamethicin was further confirmed by measuring the internuclear distances of [1-^{13}C] and [^{15}N] doubly-labeled alamethicin. Molecular dynamics simulation of alamethicin embedded into dimyristoyl phophatidylcholine (DMPC) bilayers indicates that the helical axes for α-helical N- and 3_{10}-helical C-termini are tilted 12° and 32° to the bilayer normal, respectively, which is in good agreement with the solid state NMR results.

1. Introduction

Alamethicin is an antibiotic peptide from *Trichoderma viride* that consists of 20 amino acid residues [1]. One of the major amino acid sequences is Ac-Aib-Pro-Aib-Ala-Aib-Ala-Gln-Aib-Val-Aib-Gly-Leu-Aib-Pro-Val-Aib-Aib-Glu-Gln-Phol in which 8 or 9 α-aminoisobutyric acids (Aib) are included. In addition, the N-terminus is acetylated and the C-terminus is terminated as L-phenylalaninol (Pheol) [2,3]. Alamethicin consists of heterogeneous mixture of which the major constituents which were distinctively detected by thin-layer chromatography (TLC), and named as alamethicin F30 and F50 with molar ratios of 0.85 and 0.12, respectively. Alamethicin F30 and F50 contain Glu18 and Gln18, respectively [4].

Alamethicin is known to exhibit voltage dependent ion channel activity in membrane environments [5]. Alamethicin has a high affinity for lipid bilayers, therefore, alamethicin binds to the surface of lipid bilayers and can be inserted into the membrane. The orientation of alamethicin in a lipid bilayer is dependent on the peptide/lipid (P/L) molar ratios [6,7], the type of lipid bilayers, and the membrane potentials [8]. Various channel models have been proposed to determine the ion channel activity, such as the barrel–stave model [9]. Alamethicin channels are formed by parallel bundles of the transmembrane helical monomers surrounding a central water-filled pore, and are composed of 3–12 alamethicin molecules [10,11]. The ion channel activity of alamethicin makes it a suitable model to investigate voltage dependent ion channel proteins [12,13].

X-ray crystallographic analysis indicates that alamethicin takes a helical structure where the kink position is Pro14, and the N- and C-termini take the α-helix and 3_{10}-helix structures, respectively [9]. Solution nuclear magnetic resonance (NMR) studies show that the N-terminus forms an α-helix and the C-terminus takes a variety of helix structure depending on the conditions [14–17]. In addition, circular dichroism, infrared, and Raman spectroscopies show that alamethicin

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takes a variety of helix structure in the solvents [18–21]. Furthermore, it is realized that poly-Aib takes 3_{10}-helix [22]; therefore, it is of interest to determine if the C-terminus of alamethicin may take the 3_{10}-helix structure in a membrane environment.

The conformation and orientation of membrane-bound alamethicin have been studied using solid-state NMR spectroscopy measurements with dimyristoyl phosphatidylcholine (DMPC)/dihexanoyl phosphatidylcholine (DHPC) mixed lipid bilayer systems (bicelle). ^{13}C chemical shifts of isotopically labeled alamethicin indicated that alamethicin forms an α-helical structure in a lipid bilayer and is oriented along the bilayer normal. The chemical shift anisotropy was substantially reduced by rotation of the alamethicin helix about the bilayer normal [23].

Two-dimensional separated local field ^{15}N NMR spectra were obtained using the same motional model of alamethicin where the peptides rotate about the bilayer normal. The results reveal a ^{15}N-^{1}H dipolar splitting of 17 kHz, which indicates that the N–H bond tilts 24° with respect to B_0. This angle was evaluated by assuming that the maximum dipolar splitting is 22.6 kHz and the N–H bond length is 1.024 Å [24]. These data are consistent with an α-helical conformation inserted along the bilayer normal [24].

The conformation of alamethicin in mechanically oriented phospholipid bilayers has been further studied using ^{15}N solid-state NMR in combination with molecular modeling and molecular dynamics (MD) simulations. ^{15}N-labeled variants at different positions of alamethicin along with three of Aib residues replaced by Ala were examined. From the anisotropic ^{15}N chemical shift and ^{1}H-^{15}N dipolar couplings determined for alamethicin with ^{15}N-labeling on the Ala6, Val9, and Val15 residues incorporated into phospholipid bilayers with a peptide-to-lipid molar ratio of 1:8, it was determined that alamethicin has a largely linear α-helical structure that spans the membrane with the molecular axis tilted by 10–20° relative to the bilayer normal. In particular, the compatibility with a straight α-helix was tilted by 17° and a slightly kinked molecular dynamics structure was tilted by 11° relative to the bilayer normal [25]. Measurement of the orientation-dependent ^{1}H–^{15}N dipole-dipole coupling, ^{15}N anisotropic chemical shift, and ^{2}H quadrupole coupling parameters for a single residue, combined with analysis of anisotropic interaction for the Aib8 residue provides detailed information regarding helix-tilt angle, wobbling and oscillatory rotation around the helix axis in the membrane bound state of alamethicin [26].

Alamethicin samples were uniformly labeled with ^{15}N and reconstituted into oriented palmitoyl oleoylphosphatidylcholine (POPC) and DMPC membranes. Proton–decoupled ^{15}N solid-state spectra showed that alamethicin adopts a transmembrane orientation with reconstitution into the POPC oriented membrane [27]. Two-dimensional ^{15}N chemical shift ^{1}H–^{15}N dipolar coupling solid-state NMR correlation spectroscopy (PISEMA) suggests that alamethicin in the transmembrane configuration adopts a mixed $\alpha/3_{10}$ helical structure with a tilt angle of 8.9° with respect to the bilayer normal [28].

Although, many studies show that alamethicin forms a transmembrane helix in a membrane environment, it is yet to be clarified whether the helix is bent or straight, which part of alamethicin forms the 3_{10}-helix, and whether alamethicin molecules associate with each other in the membrane environment.

We have previously reported that anisotropic chemical shift interaction in a peptide bound to membrane may exhibit chemical shift oscillation [29]. This chemical shift oscillation enables evaluation of the tilt angle of the helix and the kink angle between the two existing helices in the membrane bound peptide, as long as they rotate rapidly about the bilayer normal without using oriented membranes. From the chemical shift oscillation analysis, tilt angles with respect to the bilayer normal and phase angle of the peptides around the helical axis were determined for a variety of membrane bound peptides, such as melittin [30,31], dynorphin [32], bombolitin-II [33] and lactoferrampin [34]. In addition, it is possible to determine the existence of different types of helices within the molecule as well as the topology of the helices as was preliminarily demonstrated for alamethicin embedded in a membrane [29].

MD simulations of peptides in the membrane environment have been useful tools to investigate dynamic structure, topology and orientation with respective to membrane normal [8,35–38] and compared with those determined by solid-state NMR [25,26,33,34]. Furthermore, MD simulations provided the insight into peptide–peptide and peptide–lipid interaction networks [39].

In this study, we attempt to analyze 4 labeled sites in the N-terminus and 7 labeled sites for the C-terminus including Aib residues, which have not been labeled in the previous study. Chemical shift values for individually labeled carbonyl carbon nuclei are determined. A number of ^{13}C–^{15}N internuclear distances are also determined in order to determine the topology of alamethicin bound to membrane. The combination of chemical shift oscillation and inter-nuclear distance measurements allows to evaluation of the detailed structure, topology and orientation of alamethicin in the membrane bound state, which has not been determined in previous solid state NMR studies. MD simulations are performed to justify the accurately determined NMR structure of alamethicin in membrane environments.

2. Materials and methods

2.1. Sample preparation

The F50 amino acid sequence of alamethicin was adopted in this study and the C-terminal was methyl esterified. This modification made it possible to synthesize a number of labeled peptides with high yields. Although the C-terminus of alamethicin is not methyl ester but amino alcohol, it has been reported that this modification does not significantly affect the activity of alamethicin [24]. The sequence of alamethicin used in this study is as follows

Ac-Aib-Pro-Aib-Ala-Aib-Ala-Gln-Aib-Val-Aib-Gly-Leu-Aib-Pro-Val-Aib-Aib-Gln-Gln-PheOCH$_3$.

Eleven types of singly labeled [1-^{13}C]Ala6, Gln7, Val9, Aib10, Gly11, Leu12, Aib13, Val15, Aib16, Aib17, or Gln18-alamethicin molecules were synthesized using fluoreylmethyloxycarbonyl (Fmoc) chemistry and solid phase methods. 9-Fluorerylmethhoxycarbonyl (Fmoc)-labeled amino acids were synthesized from 9-fluorenyl N-succinimidyl carbonate (Fmoc Osu) and isotoropically labeled amino acids, following a method by Paquet [40]. A standard protocol of peptide synthesis using peptide synthesizer is described in the previous literature [41], Instead of using 1-hydroxybenzotriazole (HOBt), Fmoc-amino acids were activated using 1-[bis(dimethylamino)methylene]-1H-1,2,3-triazole[4,5-b]pyridium 3-oxid hexafluorophosphate (HATU)/dimethylformamide (DMF) in the presence of N,N-diisopropylethylamine (DIPEA), followed by amino acid coupling with stepwise cycles, because Aib has bulky sidechain to reduce the coupling activity. The synthesized peptide was treated with acetic anhydride to acetylate the N-terminus and dissolved in methanol in the presence of trifluoroacetic acid (TFA) to form the methyl ester at the C-terminus. This alamethicin analogue was purified using a Waters 600E high performance liquid chromatography (HPLC) system equipped with a Bondaphase C_{18} reversed phase column. Fifty milligrams of ^{13}C labeled alamethicins and dimyristoyl phosphatidylcholine (DMPC), with a alamethicin-to-DMPC molar ratio of 1:10, was dissolved in methanol, and solvent was subsequently evapolated *in vacuo*, followed by hydration with 600 μl of Tris buffer (20 mM Tris, 100 mM NaCl, and pH 7.5). A freeze–thaw cycle was repeated 10 times, followed by centrifugation to concentrate the bilayers. Finally, the total volume was adjusted to 300 μl containing 50 mg of lipid and alamethicin. The lipid bilayers were filled in zirconia or glass sample tube for magic angle spinning (MAS) or static NMR measurements, respectively.