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Determination of solution structure and lipid micelle location of an engineered membrane peptide by using one NMR experiment and one sample

Guangshun Wang *

The Structure-Fun Laboratory, Eppley Institute for Research in Cancer and Allied Diseases, University of Nebraska Medical Center, 986805 Nebraska Medical Center, Omaha, NE 68198-6805, USA

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Abstract

Antimicrobial peptides are universal host defense membrane-targeting molecules in a variety of life forms. Structure elucidation provides important insight into the mechanism of action. Here we present the three-dimensional structure of a membrane peptide in complex with dioctanoyl phosphatidylglycerol (D8PG) micelles determined by solution NMR spectroscopy. The model peptide, derived from the key antibacterial region of human LL-37, adopted an amphipathic helical structure based on 182 NOE-generated distance restraints and 34 chemical shift-derived angle restraints. Using the same NOESY experiment, it is also possible to delineate in detail the location of this peptide in lipid micelles via one-dimensional slice analysis of the intermolecular NOE cross peaks between the peptide and lipid. Hydrophobic aromatic side chains gave medium to strong NOE cross peaks, backbone amide protons and interfacial arginine side chain H^N protons showed weak cross peaks, and arginine side chains on the hydrophilic face yielded no cross peaks with D8PG. Such a peptide–lipid intermolecular NOE pattern indicates a surface location of the amphipathic helix on the lipid micelle. In contrast, the εH^N protons of the three arginine side chains showed more or less similar intermolecular NOE cross peaks with lipid acyl chains when the helical structure was disrupted by selective D-amino acid incorporation, providing the basis for the selective toxic effect of the peptide against bacteria but not human cells. The differences in the intermolecular NOE patterns indicate that these peptides interact with model membranes in different mechanisms. Major NMR experiments for detecting protein–lipid NOE cross peaks are discussed.

Keywords: Antimicrobial peptide; D8PG; Intermolecular NOE; Membrane peptide; NMR; Peptide–lipid interaction; Structural biology

1. Introduction

Because of the challenging nature, structural studies of membrane proteins are in the frontier of modern structural biology. Based on the mode of membrane association, these proteins are classified into two broad families: peripheral and integral. Peripheral membrane proteins bind to the surface of the membranes, while integral membrane proteins must go through the membranes. Antimicrobial peptides are ancient host defense molecules in nearly all life forms [1–4]. More than 550 mature peptides are collected in the antimicrobial peptide database [5]. Such peptides are believed to kill bacteria by targeting bacterial membranes. These peptides may be located on the membrane surface or transverse membranes. Because antimicrobial peptides usually consist of less than 50 residues, they are regarded

Abbreviations: NMR, nuclear magnetic resonance; D8PG, dioctanoyl phosphatidylglycerol; DPC, dodecylphosphocholine; DQF-COSY, double-quantum filtered correlation spectroscopy; DSS, 2,2-dimethyl-silapentane-5-sulfonate sodium salt; HSQC, heteronuclear single-quantum coherence spectroscopy; NOE, nuclear Overhauser effect; NOESY, nuclear Overhauser enhancement spectroscopy; PGs, phosphatidylglycerols; rmsd, root mean square deviation; SDS, sodium dodecylsulfate; TOCSY, total correlation spectroscopy; TOCSY-trim, a technique for identification of the key membrane-binding region of a peptide by trimming off nonessential portions based on strong TOCSY relays

* Eppley Institute, Room ECI3018, University of Nebraska Medical Center, 986805 Nebraska Medical Center, Omaha, NE 68198-6805, USA. Tel.: +1 402 559 4176; fax: +1 402 5594651.
E-mail address: gwang@unmc.edu.

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as useful models for structural studies of membrane proteins by both solution and solid-state NMR [6–10,52,53].

In humans, defensins and cathelicidins are the two major families of antimicrobial peptides. While several cathelicidins were found in animals such as sheep, cow, and pig, only one cathelicidin peptide dubbed LL-37 was identified in humans [11,12]. The importance of this host defense molecule to the health of people is now well established. Patients lacking this molecule are more susceptible to infections [13]. While cathelicidin knockout mice are more readily infected [14], expression of additional cathelicidins protects the animals from infection [15]. LL-37 also protects rats from sepsis caused by Gram-negative bacteria [16]. It is found that LL-37 is reduced in cystic fibrosis (CF) airways as a result of direct interaction with DNA and filamentous F-actin [17]. As a consequence, there is high interest in developing peptide analogs of therapeutic importance using human LL-37 as a template (reviewed in ref. [18]). While other laboratories found different active regions ranging from 20 to 24 residues [19–21], we identified a minimal antibacterial peptide corresponding to residues 17–29 of LL-37 by utilizing the TOCSY-trim experiment [22]. This 13-residue core peptide remains active after sequence reversal [23]. Of outstanding interest is that the retro-core LL-37 peptide shows sequence homology to aurein 1.2, a 13-residue antimicrobial and anticancer peptide isolated in Australian Bell frogs [24]. According to motif search of the antimicrobial peptide database [5], aurein 1.2 was found to also show sequence homology to an essential bacterial membrane anchor of enzyme IIA^{Glc} discovered in *Escherichia coli* [25]. As the membrane anchor sequence is originated from *E. coli*, it is not surprising to see that the synthetic peptide corresponding to the membrane anchor sequence of the protein is not toxic to the bacterium itself. The fact that this membrane anchor binds to anionic lipids but not zwitterionic lipids suggests a minimal membrane-targeting sequence [26]. Compared to the membrane anchor, the toxic effect of aurein 1.2 is ascribed to a more hydrophobic and longer amphipathic helix, because they have the identical number of cationic lysines. In contrast, a higher antibacterial toxicity of the retro-LL-37 core peptide, compared to the membrane anchor, results from additional cationic residues, since the two peptides have a similar hydrophobicity according to their retention times on reverse-phase HPLC [23].

The effects of ions, pH, and peptide concentration on the conformation of LL-37 were previously investigated by CD studies. At a micromolar concentration in water, LL-37 was disordered at an acidic pH but became helical with the increase in pH or salts [54]. Oren et al. [55] found that LL-37 self-associated when bound to zwitterionic lipids but disassociated into monomers in the presence of negatively charged vesicles. For structural studies of membrane-associated peptides, SDS was widely used as a mimic of anionic membranes [6,10,46,51]. We previously reported structural studies of LL-37 fragments in SDS micelles. For a C-terminal fragment of LL-37 corresponding to residues 13–37, we found that only the middle region was helical [22]. In this study, we report the three-dimensional structure of an LL-37-derived model peptide (pepA) in complex with D8PG. The rationale for the use of D8PG as a bacterial membrane-mimetic model [27–29] is that a significant population of phosphatidylglycerols (PGs) in bacterial membranes determines the selective targeting of many cationic peptides [6–10]. Based on the C-terminal segment of LL-37, pepA was designed in two steps. First, the disordered C-terminal region corresponding to residues 33–37 was removed. Second, the order of I13G14 was changed to G13I14, since many antimicrobial peptides are known to start with a glycine at the N-terminus [30]. Antibacterial assays using *E. coli* K12 found that pepA had the same antibacterial activity as full-length LL-37 (Minimal inhibition concentration 40 μM) [22]. Because neither the sequence swap nor deletion had a clear effect on the antimicrobial activity of the model peptide, pepA (sequence in Table 1) may be regarded as a useful model for human LL-37. To our knowledge, this is the first 3D structure reported for an antimicrobial peptide in D8PG. More importantly, one-dimensional peptide–lipid intermolecular NOE slice analysis also allows for the mapping of the location of the model peptide on the surface of the lipid micelle using the same NMR experiment. As a comparison, we also report the intermolecular NOE analysis of pepB, another LL-37-derived peptide with a mixture of D- and L-amino acids (sequence in Table 1). The differences in both structure and peptide–lipid NOE pattern suggest that pepA and pepB may kill bacteria in different manners.

2. Materials and methods

2.1. Materials

Both pepA and pepB (>95% pure) were synthesized and purified by Genemed Synthesis (San Francisco, CA). Protonated D8PG (>98%) was purchased from Avanti Polar Lipids (Alabaster, AL). Chloroform was removed from phospholipids under a stream of nitrogen gas followed by evaporation under vacuum overnight. All chemicals were used without further purification.

2.2. NMR spectroscopy

The NMR samples were prepared by mixing the peptide with D8PG at a molar ratio of 1:5 (2 mM peptide), containing 10% D_2O as field-locking signal. Such a ratio was found to be sufficient to stabilize the structure of the peptide. The samples were not buffered. For optimal NMR spectra, the pH of each sample was adjusted to the range of 5 to 6 by using microliter aliquots of HCl or NaOH solution and measured directly in the 5-mm NMR tube with a micro-pH electrode (Wilmad-Labglass). This is because a lower pH reduces the solubility of D8PG; a higher pH increases the exchange between peptide and water, which is unfavorable for the observation of intermolecular NOE cross peaks between arginine side chains and D8PG (below).

A two-dimensional NOESY spectrum [31] (75 ms) was recorded with 440 increments (64 scans each) in t1 and 2K complex points in t2 time domain using a spectral width of 8500 Hz in both dimensions with the ^1H carrier on water. The water signal was suppressed by WATERGATE [32]. A natural abundance

Table 1
Primary structures of PepA and PepB

Peptide	Amino acid sequence
PepA [a]	GIKEFKRIVQRIKDFLRNLV-NH_2
PepB [b]	FKRIVQRIKDFLRNLV-NH_2

[a] Corresponding to residues 13–32 of human LL-37 with the positions between I13 and G14 swapped. NH_2 means C-terminal amidation.

[b] Corresponding to residues 17–32 of human LL-37. D-amino acids are underlined.