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The structure and behavior of the NA-CATH antimicrobial peptide with liposomes

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ABSTRACT

Naja atra cathelicidin (NA-CATH) is a 34-amino acid highly cationic peptide identified in Chinese cobras to possess potent toxicity against gram-negative and gram-positive bacteria and low toxicity against host cells. Here, we report the NMR solution structure of the full-length NA-CATH peptide and its interaction with liposomes. The structure shows a well-defined α-helix between residues Phe3 to Lys23, on which one surface is lined by the side-chains of one arginine and 11 lysine residues, while the other side is populated by hydrophobic residues. The last eleven amino acids, which are predominately aromatic and hydrophobic in nature, have no defined structure. NMR data reveal that these residues do not interact with the hydrophobic residues of the helix, indicating that the C-terminal residues have random conformations. Fluorescence requenching experiments, in which liposomes serve as a mimic of the bacterial membranes, result in fluorophore leakage that is consistent with a membrane thinning or transient pore formation mechanism. NMR titration studies of the peptide–liposome interaction reveal that the peptide is in fast exchange with the liposome, consistent with the fluorescent studies. These data indicate that full length NA-CATH possesses a helical segment and unstructured C-terminal tail that disrupts the bilayer to induce leakage and lysing.

1. Introduction

Antimicrobial peptides (AMPs) are an essential component of immune systems of living organisms, and exert a direct effect on a broad spectrum of microbes including bacteria, fungi, and viruses [1–4]. They also have a wide variety of functions within the host, including immunomodulatory and chemoattractant agents [5,6]. Several AMPs have been shown to exhibit cytotoxic activity against cancer cells and have shown promise as anticancer drugs [7]. Currently, there are more than 2500 antimicrobial peptides identified and most are often less than 100 amino acids and possess numerous cationic residues, specifically lysine and arginine [1,8]. AMPs exhibit a diverse range of amino acid sequences and structural properties. They are classified into four groups based on structural themes: linear α-helical peptides, linear extended peptides (with sequences dominated by a few types of amino acids), peptides containing loop structures, and peptides with structures constrained by intra-molecular disulfide bonds [9].

Given the wide range of attributes, structures, and sequences, AMPs also exhibit various behaviors in their antibacterial role. Translocated peptides into the bacterium lead to the disruption of cellular processes, such as syntheses of nucleic acids, proteins, and cell walls [10–13] and enzymatic activity [14] to name a few [11,15]. The most common mode of action involves the disruption membrane function and integrity [16–18]. In our system, we have reported that the cathelicidin, NA-CATH, from the *Naja atra* snake, targets the membrane both in models and in cell studies [19], leading us to investigate its structure and lysing characteristics to clarify its behavior.

Within the general membrane disruption category, two primary mechanisms are hypothesized: pore formation and disruption of membrane integrity [1,9,17,20–35]. The Shai–Matsuzaki–Huang [36–39] model suggests that the amphipathic AMPs must first interact with the membrane surface electrostatically, and then insert into the membrane to form large pores [1,3,40]. In contrast, AMPs may exert its bactericidal effect with a detergent-like "carpet mechanism" [37], disrupting the structural properties of the membrane and resulting in curvature strain, membrane thinning, short lived defects, transient pores and even complete membrane dissolution [1,7,21,35,41,42]. Both mechanisms potentially lead to membrane depolarization, lipid flip-flop accompanied by AMP translocation, lipid clustering, and induced phase separation [7,43]. All of these effects are detrimental to membrane functions. In our study, we employ a combination of NMR and fluorescence requenching assays to distinguish between these two general models for NA-CATH, a highly cationic AMP.

The cathelicidin antimicrobial peptide obtained from the *Naja atra* snake, NA-CATH, displays highly potent, high efficacy behavior against gram-negative and gram-positive bacteria, and even biofilms [44] making it a promising candidate to combat resistant bacterial strains. Importantly, it exhibits minimal cytotoxic effect against host cells [45–47].

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NA-CATH has 34-amino acids and a net charge of +15, making it among the most cationic of AMPs [48]. Understanding NA-CATH structure and general behavior is critical in determining how the combination of charge, cooperativity and membrane interactions affects AMP lysing capabilities.

Previous studies reveal a semi-conserved 11 amino-acid (KRAKKFFKKLK-NH_2) motif, ATRA-1A, that has antimicrobial activity on its own, similar to the full length peptide [19]. This motif appears twice within the sequence, with only a few differences in the non-basic amino acids. Due to its length, ATRA-1A cannot span the membrane bilayer nor can it form a barrel-stave pore. Therefore, it is important to understand the structure and function of the full-length NA-CATH to distinguish possible differences in the activity of the parent and shortened peptide, as seen by Juba et al. [49].

Here, we describe the solution structure of the NA-CATH peptide by NMR spectroscopy and a mechanism of action based on fluorescence-requenching studies and titration studies by NMR. The peptide adopts an α-helical conformation between residues Phe3 and Lys23, while the remaining C-terminal 11-amino acids remain unstructured. Peptide–liposome binding studies show fast exchange between the peptide and membrane. The fluorescence and previous microscopy data give a consistent picture of liposome disruption via thinning or transient defects.

2. Experimental procedures

2.1. Materials

The peptides used in these studies were custom synthesized by GenScript (Piscataway, NJ). The supplier reported purity of NA-CATH was 95.0% based on high-performance liquid chromatography (HPLC) analysis and verified by mass spectrometry. The following lipids were purchased from Avanti Polar Lipids (Alabaster, AL) and used without modification: 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC) and 1,2-dioleoyl-sn-glycero-3-phospho-(1′-rac-glycerol) (sodium salt) (DOPG). The following materials were obtained from Invitrogen (Carlsbad, CA): 8-aminonaphthalene-1,3,6-trisulfonic Acid, disodium salt (ANTS) and p-xylene-Bis-pyridinium bromide (DPX). Sephadex® G-25 Medium gel filtration medium and 2,2,2-trifluoroethanol-d_3 (TFE or d-TFE, 99.5% purity) were purchased from Sigma Aldrich (St. Louis, MO) [22]. Heavy water or deuterium oxide (D_2O, 99.8% purity) was purchased and used without modification from Acros (Fair Lawn, NJ).

2.2. NMR spectroscopy

The NMR sample was prepared by dissolving 2.0 mg of the lyophilized peptide (NA-CATH, K^1RFKKFFKKL^{10}KNSVKKRAKK^{20}FFKKPKVIGV^{30}TFPF) into 350 μL PBS buffer consisting of 90% H_2O/10% D_2O at pH 7.4 to yield a final concentration of ~1.5 mM. The sample was placed in a 5 mm Shigemi tube. NMR data were acquired on a 600 MHz Agilent NMR spectrometer equipped with a 5-mm triple-resonance z-axis gradient probe.

An initial two-dimensional (2D) ^{1}H–^{1}H NOESY spectrum of the peptide in PBS buffer was acquired with mixing times of 100,150, and 200 msec at 21 °C. Initial evaluation of the data indicated that the peptide did not have a stable structure because there were very little ^{1}H–^{1}H correlations from the NH protons. Subsequently, the peptide sample was titrated with 2,2,2-trifluoroethanol-d_3 (TFE) and the effect on the peptide was evaluated by one-dimensional ^{1}H NMR spectrum at each 10% increment. The 2D ^{1}H–^{1}H NMR data presented here were acquired with the peptide in 30% TFE. Previously, liposomes were titrated into the peptide sample to determine whether it would stabilize the peptide structure. However, no stable structure was observed and the titration instead caused liposome lysing.

Multiple 2D ^{1}H–^{1}H NOESY spectra were acquired with mixing times of 100,150, and 200 msec. The 2D TOCSY spectrum was acquired using a DIPSI-2 pulse train with a mixing time of 15 msec. A 2D DQF-COSY spectrum was acquired. The NMR data were processed by NMRpipe [50] and analyzed with SPARKY [51] software packages.

For the structure calculation, restraints were based on NOEs observed from the NOESY spectrum acquired with 100 msec mixing time. The distance restraints used for the structure calculations were grouped into three categories of 1.8–2.8 Å, 1.8–3.3 Å, and 1.8–5.0 Å corresponding to NOE intensities of strong, medium, and weak, respectively. Restraints based on NOEs to methyl and ambiguous germinal methylene protons were assigned an additional 1.0 and 0.5 Å, respectively. For restraints involving NOEs for the aromatic side-chain protons, 2.3 Å was added to account for the pseudo-atom position for the δ- and ε-protons.

A total of 399 NOE-derived restraints were used in the structure calculations. All atoms of the peptide were included in the calculations. Structures were computed using CYANA 2.1 [52] on an iMac Macintosh computer operating with OSX 10.7. From a total of 30 randomly calculated structures, 10 were selected based on low target function values of less than one and low RMSD values. Target function values, as output by CYANA, reflect energetic influences due to violations for NOE, bond length, VdW, and angles for the calculated structure. The low target function values means that there were no violations beyond what is accepted for a well-defined structure by NMR spectroscopy. A total of 20,000 steps for torsion-angle simulated annealing were employed.

2.3. Liposome preparation

Large unilameller vesicles (LUV's, ~100 nm) were used to probe the mechanism of interaction between the NA-CATH peptide and lipid vesicles. Liposomes are historically effective to employ as a membrane mimic [53–63] and to probe protein–lipid interactions [42,54,55,58–63]. Regular liposomes, needed for the peptide–liposome interaction studies by ^{1}H NMR, and fluorophore loaded liposomes, used in the fluorescence requenching experiment were prepared as follows. Lipids dissolved in chloroform were mixed together in a ratio of 80% zwitterionic 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC) to 20% anionic 1,2-dioleoyl-sn-glycero-3-phospho-(1′-rac-glycerol) (sodium salt) (DOPG) and dried under nitrogen and under vacuum for 2 h. The vesicles were hydrated in PBS buffer (for NMR) or 10 mM HEPES/70 mM KCl buffer (for fluorescence assays) at pH 7. Loaded liposomes for fluorescence studies also contained the dye quencher pair 8-Aminonaphthalene-1,3,6-trisulfonic acid, disodium salt and p-xylene-Bis-pyridinium bromide (ANTS/DPX) at 10 mM and 15 mM respectively. The freeze/thaw method was employed to ensure adequate encapsulation of the dye and quencher. Briefly, the vesicles were allowed to swell for 5 min before being vortexed for 30 s. The vesicles were heated to 45 °C before undergoing five freeze/thaw cycles utilizing a dry ice/ethanol bath for 3 min, followed by a 45 °C water bath for 5 min. Excess dye and quencher molecules outside the loaded vesicles were removed by gel filtration using Sephadex G-25. This method produced a vesicle population of 90% LUVs (50–1000 nm) and 10% SUVs (less than 20 nm) as measured using Dynamic Light Scattering (Wyatt Technologies, Santa Barbara, CA). Studies conducted on samples of 100% SUVs do not exhibit any leakage (data not shown), therefore the presence of a small population of SUVs does not affect the leakage data. Concentration of the loaded vesicle was determined with a phosphate assay described elsewhere [64].

2.4. Peptide–liposome interaction by NMR spectroscopy

To test whether the α-helical peptide interacts with liposomes, we titrated liposomes (80:20 DOPC:DOPG) into ~1.6 mM peptide in PBS buffer with 30% TFE. The liposome was added in ~0.4 mM increments, from a 30 mM stock, and interaction with the peptide was monitored by chemical shift changes in the peptide ^{1}H signals. We also performed the liposome titration with peptide (0.25 mM) in PBS buffer without TFE.