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Stable isotope-assisted NMR characterization of interaction between lipid A and sarcotoxin IA, a cecropin-type antibacterial peptide

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ABSTRACT

Sarcotoxin IA is a 39-residue cecropin-type peptide from *Sarcophaga peregrina*. This peptide exhibits antibacterial activity against Gram-negative bacteria through its interaction with lipid A, a core component of lipopolysaccharides. To acquire detailed structural information on this specific interaction, we performed NMR analysis using bacterially expressed sarcotoxin IA analogs with ^{13}C- and ^{15}N-labeling along with lipid A-embedding micelles composed of dodecylphosphocholine. By inspecting the stable isotope-assisted NMR data, we revealed that the N-terminal segment (Leu3–Arg18) of sarcotoxin IA formed an amphiphilic α-helix upon its interaction with the aqueous micelles. Furthermore, chemical shift perturbation data indicated that the amino acid residues displayed on this α-helix were involved in the specific interaction with lipid A. On the basis of these data, we successfully identified Lys4 and Lys5 as key residues in the interaction with lipid A and the consequent antibacterial activity. Therefore, these results provide unique information for designing chemotherapeutics based on antibacterial peptide structures.

1. Introduction

Antimicrobial peptides are essential for the innate immune systems of all life forms. These peptides are supposed to kill target cells by disrupting the ordered structures of their cell membranes, resulting in the permeabilization of target cells [1]. The broad-spectrum antimicrobial activities of these peptides have potential medicinal applications [2]. In this context, detailed understanding of the structure-activity relationships of these peptides is essential for developing them into novel chemotherapeutics that can be used as substitutes for traditional antibiotics. In particular, it is considerably desirable to elucidate the modes of interaction between antimicrobial peptides and membrane components, specifically those displayed on target cells.

Lipid A is the lipophilic part of lipopolysaccharide (LPS) and is a major component of the outer membrane of Gram-negative bacteria. It consists of two glucosamine units with free phosphate groups that are linked to six acyl chains. Lipid A can be a recognition target of the complex of Toll-like receptor 4, MD-2, and CD14 in the innate immune system [3]. It can also serve as a target for antibacterial peptides [4] and C-type lectins [5].

Sarcotoxin IA is a cecropin-type antibacterial peptide produced by the larvae of the flesh fly *Sarcophaga peregrina*. It consists of 39 amino acid residues and specifically interacts with LPS and thereby exhibits antibacterial activity against Gram-negative bacteria [6]. A common feature of most insect cecropins is the presence of a tryptophan residue at position 1 or 2 and an amidated C-terminus. The N-terminal half of sarcotoxin IA is rich in positively charged or hydrophobic amino acid residues, whereas the C-terminal half is predominantly hydrophobic [6]. It was previously reported that sarcotoxin IA interacts with liposomes containing acidic phospho-

Abbreviations: CD, circular dichroism; DPC, dodecylphosphocholine; HSQC, heteronuclear single-quantum coherence; LPS, lipopolysaccharide; NMR, nuclear magnetic resonance.

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lipids, whereas its interactions with liposomes composed of phosphatidylcholine, a neutral phospholipid, were very weak [7]. Furthermore, the N-terminal dipeptide of sarcotoxin IA was demonstrated to be essential for its binding to lipid A and the consequent antibacterial activity [8].

Several attempts have been made to elucidate the molecular mechanisms responsible for LPS-specific membrane binding of sarcotoxin IA based on structural aspects. Circular dichroism (CD) data indicated that sarcotoxin IA is largely unstructured in aqueous solution, whereas it adopts an α-helical conformation under membrane–mimetic conditions [9]. ^{1}H NMR study demonstrated that sarcotoxin IA forms two discontinuous α-helices (Leu3–Gln23 and Ala28–Ala38) separated by a hinge segment in methanol solution [10]. However, no structural details are currently available for sarcotoxin IA bound to lipid A, primarily because conventional NMR analyses have been hindered by the considerable molecular sizes of their complexes in membrane–mimetic systems.

In the present study, we characterized the interaction between uniformly ^{13}C- and ^{15}N-labeled sarcotoxin IA and lipid A embedded in aqueous micelles by solution NMR spectroscopy. On the basis of chemical shift perturbation data, we successfully identified key residues involved in lipid A-binding and the consequent antibacterial activity.

2. Materials and methods

2.1. Preparation of sarcotoxin IA

Wild-type sarcotoxin IA with an amidated C-terminus was chemically synthesized by a solid phase method. Recombinant sarcotoxin IA was expressed and purified as an ubiquitin extenstion. cDNA encoding sarcotoxin IA was obtained by gene synthesis (Eurofins MWG Operon). A plasmid vector containing the sarcotoxin IA gene was constructed and cloned as a fusion protein with hexahistidine-tagged ubiquitin (His_6-Ub) using a pET28a(+) vector (Novagene), which was transformed into *Escherichia coli* strain BL21-CodonPlus (Stratagene). Transformed bacteria were grown at 37 °C in Luria–Bertani (LB) medium containing 15 μg/ml of kanamycin. To produce isotopically labeled sarcotoxin IA peptides, cells were grown in M9 minimal medium containing [^{15}N]NH_4Cl (1 g/L) and/or [U-$^{13}C_6$]glucose (2 g/L).

Protein expression was induced by adding 0.5 mM isopropyl-β_D-thiogalactopyranoside (IPTG) when absorbance reached 0.8 at 600 nm. After 4 h, cells were harvested, suspended in buffer A (50 mM Tris–HCl, 150 mM NaCl, pH 8.0) containing 4-(2-aminoethyl) benzenesulfonyl fluoride hydrochloride, and subsequently disrupted by sonication. After centrifugation, His_6-Ub-sarcotoxin IA was purified using a TALON affinity column (GE Healthcare). The expression and purification of recombinant glutathione *S*-transferase (GST)-tagged yeast ubiquitin hydrolase-1 (YUH-1) were performed as described previously [11].

Recombinant sarcotoxin IA peptide was enzymatically cleaved from His_6-Ub by incubation with GST-YUH-1 for 1 h at 37 °C at a molar ratio of His_6-Ub-sarcotoxin IA:GST-YUH1 = 10:1. Recombinant sarcotoxin IA peptide cleaved from the ubiquitin moiety was purified by reverse-phase chromatography using a C8 column (Sunniest C8, CronTech) with a linear acetonitrile gradient. The fraction containing recombinant sarcotoxin IA was collected and lyophilized.

Site-directed mutagenesis was conducted using standard PCR and genetic engineering techniques to produce sarcotoxin IA mutants. The mutations were confirmed by DNA sequencing using an ABI 3100*xl* genetic analyzer. Mutated sarcotoxin IA peptides were expressed and purified using the protocol for unmutated sarcotoxin IA peptide. Recombinant sarcotoxin IA and its mutants were dissolved at an approximate concentration of 2 mM in 0.1% (v/v) ammonia solution.

2.2. Preparation of micelles

Powdered lipid A, the diphosphoryl from *E. coli* F583, (Rd mutant) was purchased from Sigma–Aldrich. Perdeuterated dodecylphosphocoline (DPC) was purchased from Cambridge Isotope Laboratories. DPC was dissolved at a concentration of 80 mM in PBS (pH 7.0). Powdered lipid A was suspended at a concentration of 2 mM in 80 mM DPC micelle solution in PBS (pH 7.0) and vortexed.

2.3. NMR measurements

NMR spectral measurements were made using a Bruker DMX500 spectrometer equipped with a cryogenic probe. The probe temperature was set at 37 °C or 10 °C for spectral measurements of recombinant sarcotoxin IA in the presence or absence of micelles, respectively. Recombinant sarcotoxin IA that was isotopically labeled was dissolved at a concentration of 0.2 mM in PBS (pH 7.0) containing 10% (v/v) $^{2}H_2O$ with and without 8 mM DPC micelles containing 0.2 mM lipid A. NMR spectra were processed and analyzed with the program nmrPipe/Sparky. ^{1}H chemical shifts were referenced to external 2,2-dimethyl-2-silapentane-5-sulfonic acid (DSS), while ^{13}C and ^{15}N chemical shifts were indirectly referenced to DSS using the absolute frequency ratios. Secondary structural elements were identified based on the backbone chemical shifts using the programs TALOS [12] and CSI [13].

2.4. CD measurements

Sarcotoxin IA solution was prepared at 50 μM of peptide concentration in PBS (pH 7.0) with and without 2 mM DPC micelles containing 50 μM lipid A. The CD spectra of sarcotoxin IA were measured at 37 °C on a Jasco J-725 apparatus using a 1.0-mm path length quartz cell. The average of six scans was determined for each sample from which the average of blank spectra was subtracted.

2.5. Tryptophan fluorescence

Fluorescence emission spectra of sarcotoxin IA peptides were measured at 37 °C on a HITACHI F-2700 apparatus using an excitation wavelength of 280 nm and scanning from 290 to 500 nm. Sarcotoxin IA solution was prepared at 5 μM of peptide concentration in PBS (pH 7.0) with and without 0.2 mM DPC micelles containing 5 μM lipid A.

2.6. Antibacterial activity assay

The antibacterial activity of a test sample was assayed colorimetrically as described by Yajko et al. [14]. Briefly, *E. coli* BL21 cells were grown in LB medium to the middle of the exponential growth phase. Cells were placed in experimental tubes up to a concentration of 10^5 cells/ml and incubated with wild-type sarcotoxin IA and its analogs (5 nM–100 μM) for 4 h at 37 °C in the presence of an Alamar Blue solution (AbD Serotec). Bacterial growth was determined by spectrophotometry according to the manufacturer's instructions.

3. Results and discussion

For detailed characterizations of the interaction between sarcotoxin IA and lipid A, we prepared isotope-labeled sarcotoxin IA as a

recombinant peptide. For bacteria to express antibacterial peptides, their lethal effects towards host cells must be masked by protein fusion. With regard to the expression of recombinant sarcotoxin IA in *E. coli* cells, GFP, GST, and obelin have been attempted to be used as fusion partners, which could be chemically cleaved by cyanogen bromide [15]. We chose His_6-Ub as fusion partner, which could be enzymatically cleaved by YUH-1 because this strategy was successfully used to prepare isotopically labeled amyloid β peptides for NMR measurements [11,16]. By employing the His_6-Ub-fusion expression system, the final yield of sarcotoxin IA (approximately 2 mg from 1 l of cell culture in M9 minimal medium) was significantly improved as compared with yields in previous reports (e.g., final yield of approximately 0.5 mg sarcotoxin IA when bacterially expressed as a GFP-fusion in terrific broth medium) [15,17].

The released recombinant sarcotoxin IA peptide without C-terminal amidation and synthetic wild-type peptide exhibited almost identical CD and fluorescence spectra in the presence and absence of DPC micelles that contained lipid A (Supplementary Figs. 1 and 2) and were equivalent in terms of their antibacterial activity against *E. coli* (Fig. 1). These results were consistent with those in a previous report [18]. Hereafter, recombinant sarcotoxin IA lacking C-terminal amidation is simply referred to as sarcotoxin IA.

Using the isotope-labeled sarcotoxin IA, we attempted to perform heteronuclear NMR analyses to characterize its interaction with lipid A. Lipid A and lipid A/phospholipid mixtures form large aggregates, which preclude detailed NMR analyses due to severe line broadening resulting from slower molecular tumbling, although CD data indicated that sarcotoxin IA adopts an α-helical structure upon its binding to a lipid A aggregate [9]. To resolve this difficulty, we used DPC/lipid A mixed micelles as small mimics of lipid A-containing membranes.

In general, these aqueous micelles exhibit high mobility in solution, enabling high-resolution NMR spectral analyses [19]. We compared the HSQC spectra of sarcotoxin IA in the presence of DPC micelles with and without lipid A (Fig. 2A). Spectral assignments were made for the backbone chemical shifts of ^{13}C and ^{15}N as well as for ^{1}H in the free and micelle-bound states, as summarized in Supplementary Table 1.

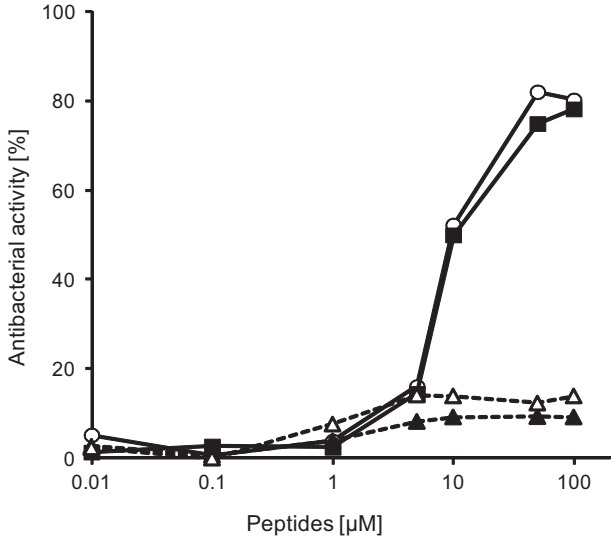

Fig. 1. Antibacterial activities of sarcotoxin IA and its mutants against *E. coli* cells. Antibacterial activity was examined using an Alamar Blue assay. The percent antibacterial activity is plotted against the peptide concentration: open circle, wild-type sarcotoxin IA with an amidated C-terminus; filled square, recombinant sarcotoxin IA lacking C-terminal amidation; open triangle, recombinant K4A mutant; and filled triangle, recombinant K5A mutant.

These spectral data revealed that sarcotoxin IA formed an α-helix at its Leu3–Arg18 segment upon binding to micelles, whereas the C-terminal segment, which forms an α-helix in methanol, was unstructured even in the presence of DPC/lipid A mixed micelles. This was consistent with the α-helix content (40%) estimated from CD data (Supplementary Fig. 1). Furthermore, lipid A embedded in micelles induced larger chemical shift perturbations $[(0.04\Delta\delta_{N^2} + \Delta\delta_{H^2})^{1/2} > 0.06$ ppm] in the peaks originating from the amino acid residues Leu3, Lys4, Lys5, Ile10, Glu11, and Gly14, which are located in the α-helix (Fig. 2B). Significant chemical shift changes were also observed for the indole NH group of Trp2.

These data indicated that the N-terminal α-helix was involved in the specific interaction with lipid A in the membrane environment. Taran et al. previously reported that an N-terminal peptide analogue (Gly1–Arg18) corresponding to the α-helix segment of sarcotoxin IA identified in the present study exhibited antibacterial activity [20].

The observed chemical shift perturbations were mapped onto the helical wheel model of the N-terminal α-helix of sarcotoxin IA, which exhibited a typical amphipathic helical structure (Fig. 2C). These results indicated that not only the residues in contact with the hydrophobic environment but also the charged residues, particularly Lys4 and Lys5 displayed on the hydrophilic face, were involved in the interaction with lipid A. Based on the NMR structure of another cecropin-like antibacterial peptide, papiliocin [21], the distance between the side-chain amino groups of Lys6 and Lys7 was estimated to be approximately 14 Å. This was intriguingly in a good agreement with the distance between the two phosphate groups of lipid A, suggesting specific electrostatic interactions between the two lysine residues.

To test the functional importance of these lysine residues, we prepared sarcotoxin IA mutants with substitutions of Lys4 or Lys5 with alanine and examined their antibacterial activities using *E. coli* cells as the target. As shown in Fig. 1, while bacterial growth was almost inhibited in the presence of 50 μM wild-type sarcotoxin IA, appreciable antibacterial activity was not detected with either the K4A or K5A mutant, indicating that these lysine residues along with the N-terminal dipeptide are indispensable for the antibacterial function of sarcotoxin IA. This was consistent with previous observations that removal of the Leu3–Lys4 dipeptide from sarcotoxin IA resulted in a 10-fold decrease in its antibacterial activities against *E. coli* [18], although this deletion may have caused a destabilization of the N-terminal α-helix [2].

Our findings provide information on the spatial arrangements of pharmacophores that are crucial for specific interactions with lipid A for designing novel chemotherapeutics based on these antibacterial peptide structures.

The present data provide new insights into the interaction between lipid A and sarcotoxin IA. However, they raise questions regarding the mechanistic processes on specific membrane environments for binding coupled with a conformational transition of antibacterial peptides, which leads to the formation of aggregates. Stopped-flow data for the interaction of sarcotoxin IA with lipid A aggregates indicated that Trp2 anchoring in the hydrophobic environment preceded α-helix formation [9]. In contrast, increasing evidence indicates that specific glycolipid clusters on cell membranes provide unique platforms for specific binding leading to abnormal aggregations of a variety of intrinsically disordered proteins associated with neurodegenerative disorders, such as amyloid β and α-synuclein [22–24]. These proteins form initial encounter complexes with the carbohydrate moieties of glycolipids and subsequently reside on the hydrophilic/hydrophobic interfaces of glycolipid clusters [11,16,25]. They ultimately undergo intermolecular interactions that lead to their aggregation. The sizes and curvatures of glycolipid clusters as well as their protein densities can be determining factors for these molecular processes.

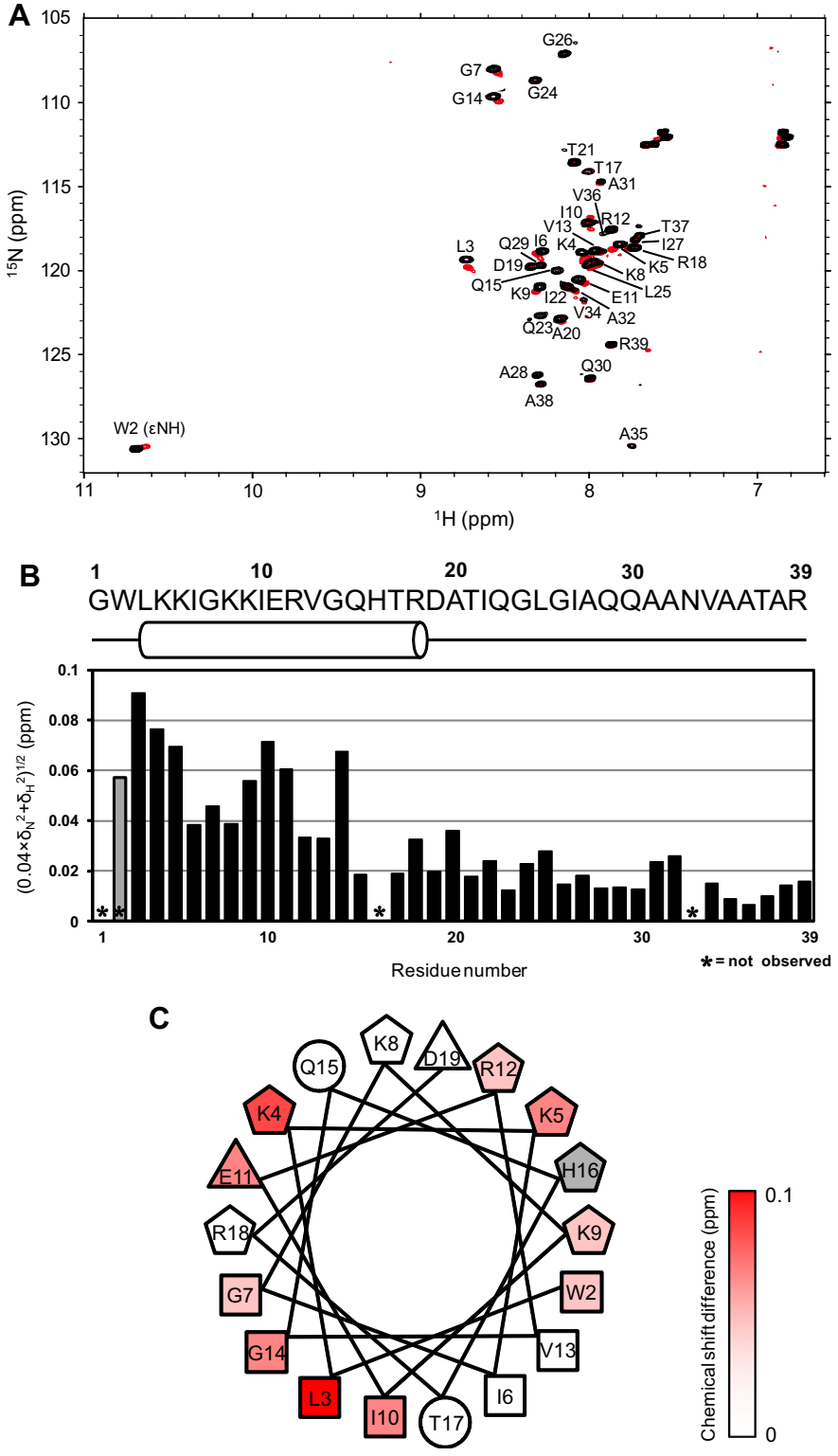

Fig. 2. (A) ^{1}H–^{15}N HSQC spectra of sarcotoxin IA in the presence of DPC micelles with (black) and without (red) lipid A. (B) Plots of the chemical shift differences of the backbone amide peaks of sarcotoxin IA in the presence of DPC micelles with and without lipid A. These data were based on the equation $(0.04\Delta\delta_{N^2} + \Delta\delta_{H^2})^{1/2}$, where $\Delta\delta_N$ and $\Delta\delta_H$ are the changes in nitrogen and proton chemical shifts, respectively. For Trp2, the indol ^{1}H–^{15}N HSQC peak was used as a spectroscopic probe (grey bar) instead of the backbone amide peak, which was not detectable. The primary structure of a sarcotoxin IA peptide with a cylinder indicating the α-helical regions is shown in the upper part of the plots. The asterisk indicates the amino acid residues that did not exhibit observable peaks in the spectrum. (C) Wheel projection of the α-helix (Trp2–Asp19) of sarcotoxin IA: circle, hydrophilic residue; square, nonpolar residue; triangle, potentially negative charged residue; and pentagon, potentially positively charged residue. The values for chemical shift differences are mapped on the wheel projection with a linear color gradient; the scale is from 0 (white) to 0.1 ppm (red). His16 was indicated with grey pentagon because its HSQC peak was not detected.

Thus, it would be interesting to delineate the similarities and differences of the molecular assembly processes in membrane environments between antibacterial peptides and neurodegenerative disorder-related proteins. Systematic NMR analyses using well-designed membrane mimics will prove to be important subjects for the forthcoming stage of this line of research, which

should provide key clues for a detailed understanding of these molecular events.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2013.01.009.

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