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Interaction between tachyplesin I, an antimicrobial peptide derived from horseshoe crab, and lipopolysaccharide

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ABSTRACT

Lipopolysaccharide (LPS) is a major constituent of the outer membrane of Gram-negative bacteria and is the very first site of interactions with antimicrobial peptides (AMPs). In order to gain better insight into the interaction between LPS and AMPs, we determined the structure of tachyplesin I (TP I), an antimicrobial peptide derived from horseshoe crab, in its bound state with LPS and proposed the complex structure of TP I and LPS using a docking program. CD and NMR measurements revealed that binding to LPS slightly extends the two β-strands of TP I and stabilizes the whole structure of TP I. The fluorescence wavelength of an intrinsic tryptophan of TP I and fluorescence quenching in the presence or absence of LPS indicated that a tryptophan residue is incorporated into the hydrophobic environment of LPS. Finally, we succeeded in proposing a structural model for the complex of TP I and LPS by using a docking program. The calculated model structure suggested that the cationic residues of TP I interact with phosphate groups and saccharides of LPS, whereas hydrophobic residues interact with the acyl chains of LPS.

1. Introduction

Gram-negative bacteria have two kinds of membranes, an inner or cytoplasmic membrane composed of phospholipid bilayer and an outer membrane. The outer membrane of Gram-negative bacteria contains lipopolysaccharide (LPS) as a major component [1]. LPS occupies more than 90% of the outer membrane [2]. LPS consists of three distinct domains: the proximal, which is a hydrophobic lipid A region consisting of acyl chains connected to two phosphorylated glucosamine residues (GlcN); the distal, which is a highly variable polysaccharide moiety called the O-antigen; and a core oligosaccharide region that is covalently linked to the other two regions [1]. LPS is well known as an endotoxin, the inflammatory properties of which cause fatal septic shock in humans [3]. Sepsis has been a serious source of mortality in many clinical cases, but no effective medical therapy has been established. To overcome sepsis, antimicrobial peptides (AMPs) that interact with LPS have recently received increasing attention in the field of drug discovery [4,5].

AMPs are ubiquitously found in living organisms [6–9] and are an important component in innate immune response. Most AMPs exhibit a wide spectrum of antimicrobial activity against Gram-negative and Gram-positive bacteria, fungi, protozoa, and viruses [10]. In general, microbes are considered to have developed little or no resistance to AMPs unlike antibiotics, which have developed marked resistance [11]. Some AMPs that interact with LPS have been reported to neutralize endotoxin [12].

When AMPs exert their antimicrobial activity against Gram-negative bacteria or neutralize endotoxin, they should interact with the outer membrane components or LPS. Structural analyses of AMPs in complex with LPS are therefore important for understanding the mechanisms underlying the action of AMPs. However, although many structures of AMPs in water have been reported, there are just a few reports on the complex structure of AMPs–LPS or the structure of AMPs in the presence of LPS [5,13,14]. Details of the interactions between AMPs and LPS and of the relationships between mechanisms of antimicrobial activity and their structures are still unclear.

Tachyplesin I (TP I) found in small granules of hemocytes of the horseshoe crab *Tachypleus tridentatus* is one of the AMPs that can bind to LPS [15,16]. TP I is a 17-residue peptide containing six cationic

Abbreviations: AMPs, antimicrobial peptides; CD, circular dichroism; DPC, dodecylphosphocholine; HPLC, high performance liquid chromatography; LPS, lipopolysaccharide; MALDI, matrix assisted laser desorption ionization; NMR, nuclear magnetic resonance; TOCSY, total correlation spectroscopy; TOF MS, time of flight mass spectroscopy; TP I, tachyplesin I; Tr-NOESY, transfer nuclear Overhauser effect spectroscopy; RMSD, root-mean-square deviation

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residues and two disulfide bonds (C3–C16 and C7–C12), and its C-terminus is amidated [15]. In water, TP I forms an antiparallel β-sheet structure [17,18]. The conformations of TP I in the presence of DPC micelles were also determined [18]. Furthermore, TP I mutants have been studied intensively. The structure of TPY4, a TP I mutant in which all the Cys residues are replaced with Tyr, has been revealed in water and in the presence of DPC micelles [18]. CDT is a mutant of TP I in which all Cys residues are deleted, and its LPS-bound structure has been determined [19]. However, the complex structure between wild-type TP I and LPS remains unclear. In order to obtain detailed structural information about the interaction between TP I and LPS, we investigated the structure of TP I in the presence of LPS by circular dichroism (CD) measurements, fluorescence experiments, nuclear magnetic resonance (NMR) measurements and docking calculation.

2. Materials and methods

2.1. Peptide and reagents

TP I (KWCFRVCYRGICYRRCR-NH_2) was synthesized by Fmoc solid phase chemistry (Sigma Life Science). Based on the protocols of Powers et al. [20], we oxidized and purified TP I. In brief, TP I was dissolved in 20 mM Tris–HCl buffer (pH 8.0) and the solution was shaken for more than 24 h at room temperature to promote disulfide bond formation by oxidation. The correctly folded TP I (C3-C16 and C7-C12) was then separated and purified by reverse-phase HPLC. Two disulfide bonds of the purified peptide were confirmed by MALDI-TOF mass spectroscopy through an observed 4 mass unit difference between the reduced and oxidized forms of TP I. Oxidized TP I solution was lyophilized and stored at −80 °C. LPS of *Escherichia coli* O111:B4 was purchased from Sigma-Aldrich and was used without any further purification in all experiments.

2.2. Circular dichroism spectroscopy

CD spectra were recorded on a model J-725 spectropolarimeter (Jasco) using a quartz cell with a 1 mm path length. Spectra were measured at 25 °C over the range from 190 to 250 nm at a scanning speed of 50 nm/min. TP I and LPS were dissolved in 10 mM sodium phosphate buffer (pH 7.4) and the concentrations were 30 μM and 72 μM, respectively. The contribution from the buffer was removed by subtracting the spectrum of a sample without TP I.

2.3. NMR spectroscopy

All NMR spectra were recorded either on a BRUKER DMX 500 MHz equipped with a cryo-probe or a JEOL ECA 600 MHz spectrometer. Data were processed using NMRPipe 4.1 and NMRDraw 2.3 [21] and analyzed using Sparky 3.113 software [22]. ^{1}H NMR experiments were performed at 35 °C with a TP I concentration of 750 μM in an aqueous solution containing 50 μM LPS and 10% D_2O, pH 3.8. The spectra of TP I in free solution were acquired at 1 mM peptide concentration. The mixing times of two-dimensional TOCSY [23] and Tr-NOESY [24] spectra were 90 and 150 ms, respectively. The interaction of TP I with LPS was examined by measurements of 1D ^{31}P NMR at 35 °C. LPS (0.4 mM) in 10% D_2O at pH 3.8 was titrated using TP I at concentrations of 0.2, 0.4, and 0.6 mM. We confirmed that no oligomerization of the peptide is present under our experimental conditions (data not shown).

2.4. Calculation of the three-dimensional structure of TP I

In the presence and absence of LPS, a total of 216 and 110 distance restraints were acquired from the Tr-NOESY and NOESY spectra, respectively. The TP I structures with or without LPS micelles were calculated using XPLOR-NIH [25,26]. A total of 100 structures were calculated and the quality of the structures was checked with the program PROCHECK-NMR [27] for the 20 lowest-energy structures. Structures were visualized using PyMOL [28]. NMR resonance assignments for TP I in LPS and in water have been deposited in the BioMagResBank (BMRB) entry 11538 and 11539, respectively. The structural coordinates of TP I in LPS and in water have been deposited in the Protein Data Bank (PDB) ID 2MDB and 2RTV, respectively.

2.5. Tryptophan fluorescence studies

All fluorescence experiments were carried out using a 650-40 fluorescence spectrophotometer (Hitachi) and 1 cm path length cuvette.

TP I was dissolved in 10 mM sodium phosphate buffer (pH 7.4) and its concentration was 5 μM. The intrinsic tryptophan fluorescence emission spectra were obtained by titrating a stock solution of 1 mM LPS into a TP I solution. The exciting wavelength was set to 280 nm and emission was monitored from 300 to 500 nm at 25 °C.

Fluorescence quenching experiments were performed by stepwise addition of acrylamide from a stock solution of 5 M into sample solution at 25 °C. The sample solution contained 5 μM TP I and 12 μM LPS in sodium phosphate buffer at pH 7.4.

The results of the fluorescence quenching experiments were analyzed by fitting to the Stern–Volmer equation, $F_0 / F = 1 + Ksv[Q]$. F_0 and F are the fluorescence intensities at the appropriate emission wavelength in the absence and presence of acrylamide, respectively. Ksv is the Stern–Volmer quenching constant and $[Q]$ is the molar concentration of acrylamide.

2.6. Docking of TP I with LPS

The structure of TP I calculated from the distance restraints of Tr-NOE was docked onto LPS by the program AutoDock 4.2 [29]. Docking calculations were carried out based on the protocols of Bhunia et al. [13]. The atomic coordinate of LPS (PDB ID 1QFG) used for docking calculations was obtained from the co-crystal structure of LPS and Fhu A [30]. The TP I was used as a ligand and backbone was kept rigid, whereas almost all side chains were defined as flexible. LPS was defined as a receptor and was kept rigid. Grid maps representing LPS were constructed using 70 × 80 × 80 points, with a grid spacing of 0.375 Å and grid center of the H2 atom of the GlcN II in lipid A. Docking calculation was carried out by using a Lamarchian genetic algorithm (LGA) with a translation step of 0.2 Å, a quaternion step of 5°, and a torsion step of 5°. The maximum number of energy evaluations increased to 15,000,000. Two hundred LGA docking runs were performed.

3. Results

3.1. Circular dichroism spectroscopy

We measured CD spectra of TP I in the presence or absence of LPS to estimate the secondary structures of TP I, and investigated whether TP I changes its conformation by binding to LPS. First, we measured CD spectra of TP I in the presence and absence of LPS at temperatures ranging from 25 °C to 40 °C and confirmed that there were no changes in the spectra (data not shown). The horseshoe crab is a heterothermic, saltwater animal, so we adopted the result at 25 °C. The spectrum of TP I in water displayed two positive bands at 198 and 232 nm and one negative band at 211 nm (Fig. 1). These bands are indicative of a β-sheet structure and a β-turn structure. This estimated structure of TP I in water is consistent with a previous report [16]. TP I in the presence of LPS also exhibited two positive bands at 204 and 232 nm and one negative band at 212 nm (Fig. 1). This spectrum indicated that TP I retains a β-sheet structure and a β-turn structure in its LPS-bound state. If none of the conformational changes are caused by binding to LPS, the spectrum of TP I fits with that of TP I in the presence of LPS. As is obvious from the spectra, the two spectra do not completely fit each other. This suggested that TP I changes its conformation by binding to LPS.