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Brief Article

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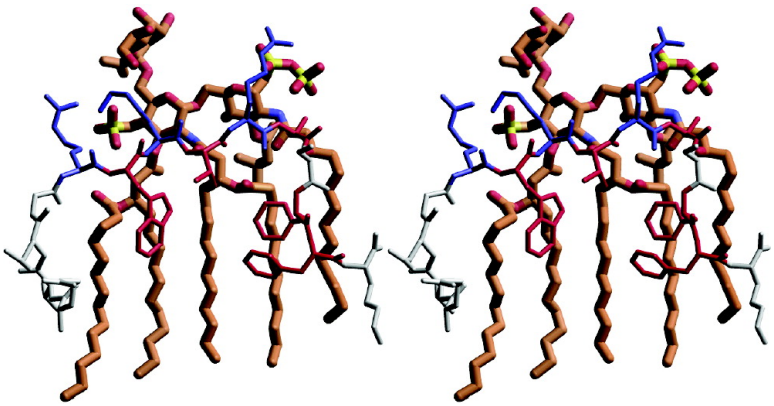

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Structure of a Synthetic Fragment of the Lipopolysaccharide (LPS) Binding Protein When Bound to LPS and Design of a Peptidic LPS Inhibitor

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Peptidic lipopolysaccharide (LPS) antagonists are the subject of intensive research. We report an NMR and modeling study of LBP-14 (RVQGRWKVRASFFK), a synthetic fragment of the LPS binding protein (LBP). In a mixture with LPS we observed the transferred nuclear Overhauser effect and determined the LPS-bound structure of LBP-14 that was used for docking calculations to LPS. The derived complex was used to design a peptide that displayed more than 50% increase in LPS inhibition in vitro.

Introduction

Lipopolysaccharide (LPS, or endotoxin) induces Gram-negative sepsis, and septic shock remains lethal in up to 60% of cases.[1] LPS antagonists that neutralize its endotoxic action are the subject of intensive research. LPS is the main constituent of the outer membrane of Gram-negative bacteria. Structurally it is an amphiphile consisting of lipid A (see Supporting Information), a core oligosaccharide, and an O-specific chain. The lipid A moiety is responsible for endotoxic activity and is the most highly conserved part of the structure, typically with two glucosamines, two phosphate esters, and five to seven fatty acids.[2] LPS in serum initially binds with high affinity to the LPS binding protein (LBP), a 60 kDa serum glycoprotein synthesized in hepatocytes.[3] LBP catalyzes the transfer of LPS to membrane-bound CD14 (mCD14) on monocytes or monocytically derived cells. Final transmembrane signaling takes place when mCD14 next transfers or presents LPS to the transmembrane toll-like receptor-4 (TLR4) protein that works in conjunction with an obligate accessory protein MD-2, forming a TLR4/MD-2 complex that initiates intracellular signaling and release of a wide spectrum of cytokines and cellular mediators (see ref 4 and references therein).

Recent developments in identifying novel strategies that overcome endotoxic shock involve LPS-neutralizing peptides that are derived from natural antimicrobial peptides or from proteins that bind LPS,[5] despite known difficulties with rapid degradation of peptides in the organism. On the basis of sequence alignment with the positively charged amphipathic hairpin loop of the limulus anti-LPS factor (LALF,[6] residues 32−50) probably containing the LPS binding site, an analogous loop (residues 86−104) was proposed for LBP and the bactericidal/permeability increasing protein (BPI), a 55 kDa protein causing endotoxin neutralization and, contrary to LBP, bacterial killing. Peptides with limited LPS neutralizing capacity involving residues 82−108 of LBP and BPI were synthesized.[7,8] The peptide corresponding to residues 86−99 of LBP that was reported to retain significant binding to LPS and inhibit binding of LPS to LBP was subjected to Ala scanning to identify amino acids essential for LPS binding,[9] while Trp91 and Lys92 were indispensable for peptide−LPS interactions and inhibition of LBP−LPS binding the mutant Lys95 (LBP-14; residues 86−99 with Lys95 replaced by Ala, RVQGRWKVRASFFK) was the most active in blocking LPS binding to LBP.

In our lab we determine structures of peptides in complex with LPS[10−12] using the transferred nuclear Overhauser effect (TRNOE)[13] that occurs in the case of weak binding of a smaller ligand (in the present case, peptide) to a large molecule or assembly (in the present case, LPS aggregate). The intramolecular NOEs of the bound ligand occur at the resonance frequencies of the free ligand and allow the determination of the bound-state conformation of the peptide from the intramolecular NOE interactions between ligand protons. In this paper we present spectroscopic and computational results of LBP-14.[9] In a mixture with LPS we observed the TRNOE and could determine the bound conformation of the peptide. We used molecular modeling to derive complexes of LBP-14 and LPS that served as a basis for rational design of a peptide with enhanced antiendotoxic properties. The peptides were tested in vitro for biological activity; the novel peptide displayed more than 50% increase in LPS inhibition in vitro.

Results

Free LBP-14 in Water Solution. NMR. The spin systems in the total correlation spectra of LBP-14 are fairly well resolved at 25 °C and allow complete assignment of proton resonances using $d_{\alpha N}(i,i+1)$ and $d_{\beta N}(i,i+1)$ connectivities in the NOESY spectra.[14] The NOE and ROE patterns contain only intraresidual and sequential connectivities and are indicative of a highly flexible molecule (see Supporting Information).

LBP-14 Bound to LPS. NMR. The addition of LPS to the aqueous solutions of LBP-14 to give peptide/LPS of 10:1 w/w leads to a large increase in number and

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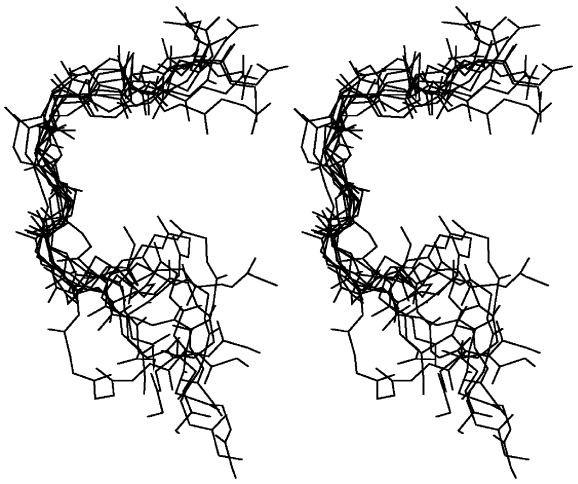

Figure 1. Stereoview of the ensemble of NMR refined LBP-14 structures (backbone rmsd at residues 6−13, 1.71 ± 0.39 Å) bound to LPS as determined by the TRNOE experiment.

intensity of the signals in the NOE spectra. A number of long-range NOE connectivities involving the hydrophobic residues appears, from Val8 H^{γ} to Trp6 $H^{\epsilon 1}$, Trp6 $H^{\epsilon 3}$, Phe12 H^{δ}, and Phe13 H^{ϵ} and from Ala10 H^{β} to Phe12 H^{ϵ} (see Supporting Information). None of the mentioned NOEs are observed in the free peptide. The only hydrophobic residue that is not involved in any long-range connnectivity is Val2.

NMR Structure Refinement. The number of meaningful NOE-derived distances used for the torsion angle dynamics of LBP-14 was 39. The best 10 structures of LBP-14 with the lowest target function after the energy minimization protocol display a sufficiently well-defined family of conformations in residues 6−13 (rmsd superposition at the backbone atoms, 1.71 ± 0.39 Å; Figure 1). None of the residues are found in forbidden regions of the Ramachandran plot (data not shown). The backbone in residues 6−13 forms an almost planar hairpin-like structure surrounding a hydrophobic core consisting of Trp6, Val8, Phe12, and Phe13, making the structure highly amphipathic.

Docking. In molecular docking calculations, 5 peptide structures were docked to LPS, each producing 20 docked structures of which 10 with low final docked energy were analyzed. Not all structures converged to the same binding mode; however, for all five peptide structures the ones with the lowest final docked energy showed the same binding mode (Figure 2).

Identification of LBP-14 Residues That Interact with LPS. The residues of LBP-14 that interact with LPS in the best docking model (Figure 2) are listed in Figure 3A. Arg9 and Arg5/Lys7 are involved in electrostatic contacts with the phosphate groups of GlcN I and GlcN II, respectively, while Trp6, Val8, Ala10, and Phe12 (and probably Phe13) are involved in hydrophobic contacts with the acyl chains of LPS. The N-terminal residues Arg1−Gly4 do not show a consistent pattern of interaction with LPS.

Design of a Peptide with Improved Interaction with LPS. The changes introduced with the novel peptide are depicted in the last row of Figure 3A. Hydrophobic contacts according to the binding model were enhanced by replacing both Val residues with Leu, Ala10 with Phe, and Phe13 with Trp. Solubility of the

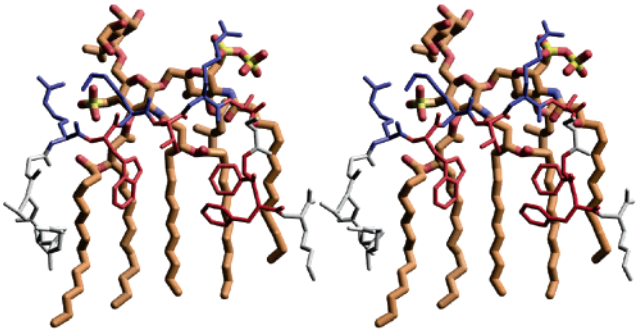

Figure 2. Structure of the LBP-14/LPS complex calculated with AutoDock.[25] The LPS structure is from ref 24. The basic residues are drawn blue, and the hydrophobic residues are shown in red. Only heavy atoms are displayed for clarity.

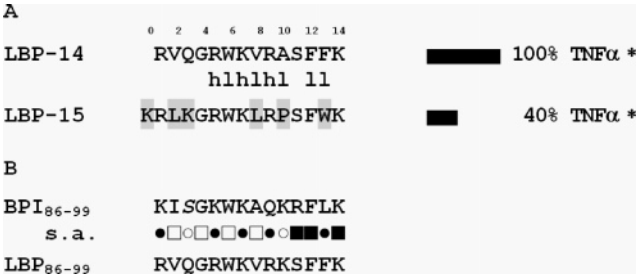

Figure 3. (A) Residues of LBP-14 (first row, one-letter code) that, according to the best docking model (Figure 2), interact with LPS (second row, (h) hydrophillic, (l) lipophillic). The changes introduced in the novel peptide LBP-15 are depicted in the third row (shaded). The in vitro TNFα response of purified human PBMC to LPS in the presence of the peptide is shown in the last column (∗, value relative to LBP-14; small value means better inhibition). (B) Aligned fragments 86−99 of BPI and LPS (one-letter code). The residue solvent accessibilities (s.a., (□) <10%, (○) <30%, (●) >30%, (■) >60%) of BPI_{86-99} in the full protein are shown in the second row.

peptide was preserved by introducing a Lys residue at the N-terminus and another in place of Gln3. In NOE spectra of the novel peptide in mixture with LPS, no TRNOE was observed.

LPS Inhibition in Vitro. Both peptides were subjected to measurements of LPS inhibition in vitro. The LBP-14 peptide displays only moderate inhibition of TNFα formation in buffy coat cells upon challenge with LPS, comparable to the parallel plate with no added peptide. The amount of TNFα formed was set to 100% (Figure 3A, right). In the same experiment with LBP-15 the amount of TNFα measured was significantly less (40% relative to LBP-14; Figure 3A, right), proving increased LPS inhibition.

Discussion

The design of antiendotoxic drugs has proved to be very difficult. A prerequisite for contemporary rational drug design is a thorough understanding of LPS−peptide recognition at the atomic level. LBP shares 45% sequence identity with BPI whose structure is known (ref 15, PDB code 1BP1); both proteins can be aligned without interruption from residue 1 to residue 123.[15] The sequence identity in residues 86−99 is 43% (Figure 3B); allowing for Lys ↔ Arg, Ile ↔ Val, Ala ↔ Val, and Leu ↔ Phe substitutions, the sequence identity becomes almost 80%. The fragment BPI_{86-99} is situated in a

β-hairpin located at the tip of the N-terminal barrel, with residues 96 and 97 forming a type II′ β turn, thus making the hydrophobic residues Phe87 and Leu88, corresponding to Phe12 and Phe13 in LBP-14, highly solvent accessible (Figure 3B, second row). Assuming that the fragment LBP_{86-99} adopts a very similar conformation in the full LBP protein as BPI_{86-99} does in BPI, the same would be true for LBP-14, with residues Ser11 and Phe12 forming the β turn. Such a conformation is not induced upon contact with LPS. The structure of the isolated peptide fragment is therefore not the same as in the intact protein, even if it interacts with the same ligand (LPS) in both cases. The same has been observed in the case of lactoferricin.[16]

Also for this reason, the TRNOE method to determine the three-dimensional structures of peptides bound to proteins, or other macromolecular systems, is becoming increasingly important in drug design efforts.[17] In this work we show that even difficult systems such as peptides interacting with LPS aggregates can provide sufficient structural data. The experimental studies of antiseptic peptides in complex with LPS using NMR are difficult because of the amphiphillic nature of the peptides that tend to aggregate at concentrations necessary for measurement. Additionally, conditions for the TRNOE effect are not always attainable. The latter is measured in approximately 20:1 peptide/LPS molar ratio, so no experimental information is obtained on the receptor (i.e., LPS) structure or its contact points with the peptide. The latter could be obtained using saturation transfer difference (STD) experiments.[18] The latter, however, has not yet been, to the best of our knowledge, successful with LPS. The structures of the complexes therefore have to be modeled[19] using the experimentally obtained bound conformation of the peptide and a suitable structure of the receptor. In recent years computer modeling of protein−ligand interactions (docking) has become a valuable tool for fast computer-based screening and single docking experiments.[20,21] While predictions of the structures of complexes between rigid ligands and protein targets have become very reliable, the number of possible solutions with flexible ligands, e.g., peptides, remains high. The set of possible solutions may be greatly narrowed by knowledge of the bound conformation.

The latter in the case of LBP-14 interacting with LPS is amphipathic and binds with an electrostatic and a hydrophobic component. Both may be used to predict changes in the peptide that would enhance binding (Figure 3A). In the present case the novel peptide LBP-15 shows enhanced binding to LPS (the interaction leads to formation of aggregates upon titration of the peptide with LPS that is visible as precipitate; also, the absence of the TRNOE indicates stronger binding) and enhanced LPS inhibition in vitro. Although the changes introduced into LBP-15 may have changed the mode of binding and the inhibition was measured at 50 μM peptide, these results indicate that rational drug design based on TRNOE data may provide clues for the development of new immunomodulatory agents for use as therapy in the treatment of Gram-negative bacterial sepsis. In future work step-by-step point mutations will be employed to enhance the characteristics of the peptide.

Experimental Section

NMR. LBP-14 and LBP-15 were synthesized at the Keck Biotechnology Resource Laboratory, Yale University (purity >95%). The NMR spectra were obtained in 95% H_2O and 5% D_2O solution at 298 K and at 1−2 mM peptide. All experiments were performed at pH 4.5 using a 20 mM phosphate buffer. LPS from *E. coli*, serotype 055:B5, was obtained from Fluka. The concentrations of LPS are given in mass per volume because of the heterogeneity of the polysaccharide outer core.

The assignment of ^{1}H resonances was performed using standard TOCSY (mixing times of 10 and 70 ms) and two-dimensional NOESY and ROESY (mixing times of 80−200 ms) on a Varian Inova 600 spectrometer and a Bruker DMX 600 spectrometer. Water signal suppression was achieved using presaturation or WATERGATE. Two-dimensional NOESY experiments with mixing times of 80−150 ms were carried out using mixtures of LBP-14 and LPS that correspond to 10:1 w/w ratios. A NOESY with a mixing time of 80 ms was used for structure determination.

Computational Methods. One-hundred different structures were calculated using the torsion angle dynamics program DYANA.[22] Ten structures selected according to the lowest target function were energy-minimized, including distance restraints using the DISCOVER program (Accelrys, San Diego, CA) with the consistent valence force field,[23] on a Silicon Graphics O2 workstation. The side chains were kept uncharged during minimization to avoid charge repulsive effects. A distance-dependent dielectric constant was employed to simulate, at least in part, electrostatic screening by the solvent.

The coordinates of the lipid A portion of LPS from ref 24 (including two KDO residues) and the LBP-14 coordinates of the five structures with the lowest target function from NMR refinement were used for molecular docking calculations with the program AutoDock.[25] The peptide backbone was kept rigid using the data from the NOE experiments, while all side chains were defined as flexible using the deftors module. Lipid A was treated as the macromolecule part of the docking calculation and was kept rigid. The AutoGrid calculation was run with 100 points (separated by 0.325 Å) in each spatial dimension, with the grid centered at the H2 atom of the GlcN II residue of the lipid A moiety. The AutoDock calculation was run using the Lamarckian genetic algorithm using a translation step (tstep) of 0.2 Å, a quaternion step (qstep) of 5.0° and a torsion step (tstep) of 5.0°, producing 20 structures for each starting structure. In the analysis step, 10 of the generated docked structures were evaluated.

In Vitro LPS Inhibiton.[26] Human peripheral blood mononuclear cells (PBMC) from the buffy coat of healthy blood donors were isolated by density gradient centrifugation with Ficoll-Paque (Pharmacia, Sweden). The cells were cultured in RPMI 1640 cell culture medium (Sigma, Germany) supplemented with 100 μg/mL penicillin (Sigma), 100 μg/mL streptomycin (Sigma), 2 mM L-glutamine (Sigma), 20 mM Hepes (Sigma), and 5% heat-inactivated AB normal human serum (Sigma). The peptides were dissolved in RPMI 1640 at 50 μM and tested for their ability to neutralize LPS. The latter was prepared as a 10 ng/mL dilution in cell culture medium; so was polymyxin B (10 μg/mL) as an established LPS-neutralizing compound. The 1×10^6 cells (final culture volume, 1.5 mL) were plated in 24-well culture plates (Costar) with each peptide solely or a combination of a peptide and LPS preincubated for 1 h at 37 °C or a combination of polymyxin B and LPS preincubated for 1 h at 37 °C or LPS or medium solely, at 37 °C in a humidified atmosphere of 5% CO_2 in air. The cell-free supernatants were collected at 4 h intervals and stored at −70 °C before being evaluated for TNF-α by the commercially available ELISA kit (Milenia Biotec, Germany).

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Appendix

Abbreviations. BPI, bactericidal/permeability increasing protein; GlcN, glucosamine; TRNOE, transferred nuclear Overhauser effect; KDO, 3-deoxy-D-manno-2-octulosonate; LBP, LPS binding protein; LPS, lipopolysaccharide; NOESY, nuclear Overhauser effect spectroscopy; ROESY, rotating-frame Overhauser enhancement spectroscopy; rmsd, root-mean-square deviation; TNF-α, tumor necrosis factor α; TOCSY, total correlation spectroscopy.

Supporting Information Available: Table of resonance assignments, chemical structure of a typical lipid A from *E. coli*, and part of the NOE spectrum of LBP-14, isolated and in mixture with LPS. This material is available free of charge via the Internet at http://pubs.acs.org.

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