Brief Articles

Structure of a Synthetic Fragment of the LALF Protein When Bound to Lipopolysaccharide

Primož Pristovšek,*,† Krisztina Fehér,‡ László Szilágyi,‡ and Jurka Kidrič†

Laboratory of Biotechnology, National Institute of Chemistry, Hajdrihova 19, SI-1000 Ljubljana, Slovenia, and Department of Organic Chemistry, University of Debrecen, Faculty of Science, Egyetem tér 1, H-4010 Debrecen, Hungary

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Peptidic lipopolysaccharide (LPS) antagonists are the subject of intensive research. We report an NMR and modeling study of LALF-14 (GCKPTFRRLKWKYKCG), a synthetic cyclized fragment of the limulus anti-LPS factor (LALF) comprising residues 36−47. In a mixture with LPS we observed the transferred NOE effect and derived the LPS-bound structure of LALF-14. Neither the free nor the LPS-bound peptide displays NOEs indicative of a β-sheet-like structure that is adopted by the fragment in the full-size protein. However, docking calculations show that the former structure is not a prerequisite for binding of LALF-14 to LPS.

Introduction

Very low concentrations of lipopolysaccharide (LPS) are required to incite septic shock, a multiple organ dysfunction syndrome that is associated with high mortality in intensive care unit patients.[1] LPS, the main constituent of the outer membrane of Gram-negative bacteria, is an amphiphile, consisting of the lipid A, a core oligosaccharide, and an O-specific chain. The core region contains several sugars, among them 3-deoxy-D-manno-2-octulosonate (KDO), unique to LPS. The lipid A moiety is responsible for the 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] (see Supporting Information).

Recent developments in identifying novel strategies to overcome endotoxic shock involve LPS-neutralizing peptides that are either derived from natural antimicrobial peptides or from proteins that bind LPS[3] despite of known difficulties with rapid degradation of peptides in the organism. The design of antiseptic peptides has proved very difficult. A prerequisite for contemporary rational drug design is a thorough understanding of LPS−peptide recognition at atomic level. The interaction of LPS with its cognate binding proteins has been structurally elucidated in the single case of the X-ray crystallographic structure of LPS in complex with FhuA from *E. coli* K-12 (PDB entry 1QFF[4]). Four positively charged residues of FhuA were identified that appear to provide most of the important electrostatic interactions with LPS.[5] Their arrangement was proposed as a common LPS binding motif and was used to identify LPS-binding sites on LPS binding proteins with known 3D structure, e.g., the limulus anti-LPS factor (LALF[6]). The latter, a small (101 amino acids) basic protein that binds and neutralizes LPS, displays a single domain consisting of three α-helices packed against a four-stranded β-sheet. Strands 2 and 3 of the β-sheet run the length of the protein, forming a positively charged amphipathic hairpin loop with a β-turn (residues 32−50). This loop was proposed to involve the binding site for LPS.

Conformational studies of antiseptic and/or antimicrobial peptides in several solvents do not yield a convincing bioactive conformation.[7] The use of experimental NMR methods, most prominently the exchange-transferred nuclear Overhauser effect spectroscopy (etNOE),[8] is able to overcome this limitation. The etNOE occurs in 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 way we have determined the LPS-bound structure of polymyxin B (PmB), a cyclic, cationic, high affinity endotoxin-binding peptide antibiotic, and proposed a model of the complex.[9] However, a refined picture of the interactions remained desirable since the determination of the thermodynamic properties of LPS−PmB complexes using calorimetric titration indicates that complexation is entropically driven.[10]

We studied the LPS-bound conformations of anti-endotoxic peptides with LPS using the etNOE[11,12] and derived models of complexes with LPS using molecular docking protocols.[13] In this paper we present spectroscopic and computational results of a synthetic 16-residue fragment of LALF (residues 36−47 with a synthetic disulfide bridge, GCKPTFRRLKWKYKCG, LALF-14[14]) that was reported to have a specific lipid A binding activity comparable to the PmB. In the present work, we observed the etNOE in a mixture

* Corresponding author. Tel./Fax: +386 1 4760 377/300; e-mail: primoz.pristovsek@ki.si.
† National Institute of Chemistry.
‡ University of Debrecen.

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with LPS and could determine the bound conformation of the peptide. The arrangement of the side-chains makes the molecule highly amphipathic and allows strong electrostatic and hydrophobic contacts with LPS that are different from the ones in the full-size protein.

Results

Free Peptide in Water Solution. NMR. The spin systems in the TOCSY spectra of LALF-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[15] (see Supporting Information). Only a few cross-peaks are present; the peptides at 25 °C and 600 MHz proton frequency are found in the negative NOE regime close to the zero region. The NOE and ROE patterns contain only intraresidual and sequential connectivities (data not shown) and are indicative of a highly flexible molecule. No NOEs indicating a β-sheet like structure, with the β-turn at residues 41 and 42 that is adopted by the fragment in the full-size protein, are observed.

Peptide Bound to LPS. NMR. The addition of LPS to the aqueous solutions of LALF-14 leads to moderate line broadening at weight ratios of peptide:LPS 11:1 (data not shown). The chemical shifts of the resonances remain unchanged upon addition of the LPS stock solution. A more dramatic effect is observed in the NOESY spectra of the mixtures that display a large increase in number and intensity of the signals. A number of long-range NOE cross-peaks involving the residues on opposite sides of the disulfide-bridged cycle appears, most prominently the NOEs Pro37 β−Tyr46 ϵ, Thr38 HN−Tyr46 β, Thr38 HN−Tyr46 δ, and Thr38 γ2−Tyr46 δ (see Supporting Information). Additionally, the medium-range NOE Arg41 α−Lys43 HN is observed in the region of the turn (residues 40−43), along with the sequential NOE Leu42 HN−Lys43 HN; the latter NOE pattern is consistent with a type-II β-turn at residues 41−42. Other medium-range NOEs are observed between the side-chains of Leu42 and Trp44, most prominently Leu42 δ−Trp44 ϵ1, that indicate hydrophobic contacts between the two residues. Nearly none of the mentioned NOE peaks are observed in the free peptide.

Molecular Modeling. NMR Refinement. The number of meaningful NOE-derived distances used for the torsion angle dynamics of LALF-14 was 77. The best five structures of LALF-14 with lowest target function after the energy minimization protocol (for coordinates see Supporting Information) display a sufficiently well-defined family of conformations (RMSD superposition at the backbone atoms of residues 35−48 2.17 ± 0.40 Å; Figure 1A). None of the residues is found in forbidden regions of the Ramachandran plot (data not shown). No regular secondary structure elements are observed in any part of the structure. The backbone forms a hairpin-like structure with a loop at residues 38 and 44; the type-II β-turn at residues 41−42 suggested by the NOE pattern mentioned above is not observed in the calculated structures, confirming that the NOE pattern is a necessary but not sufficient condition for a type-II β-turn. The backbone is almost planar with side-chains being directed below or above the plane in an alternating fashion. Viewing the peptide backbone in its horizontal plane with Gly34 in front on the left and Gly49 in front on the right places the S−S bridge between Cys35 and Cys48 below the backbone plane, along with the side-chains of Pro37, Phe39, Trp44, and Tyr46, while the ones of Lys36, Thr38, Arg40, Lys43, Lys45, and Lys47 are placed above the plane (Figure 1B). The ideal pattern of alternating residues is broken at the position of Leu42 that has its side-chain placed above the plane but distorts the backbone in order to find proximity with the side-chain of Trp44, as indicated by numerous NOE connectivities between the side-chains of Leu42 and Trp44. The resulting two faces of the planar peptide are therefore highly amphipathic, the lower side being hydrophobic and the upper one highly positively charged.

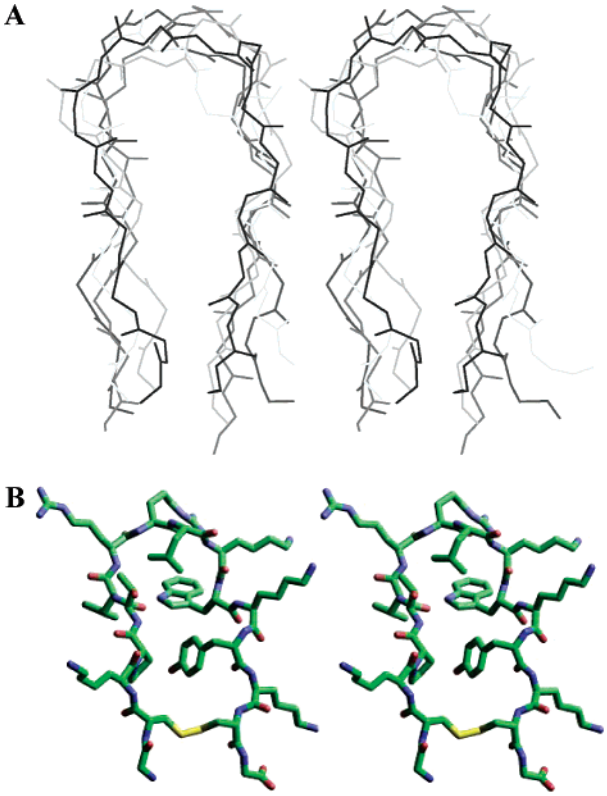

Figure 1. Stereoview of (A) the ensemble of NMR refined LALF-14 structures (backbone RMSD at residues 35−48 2.17 ± 0.40 Å) and (B) a representative structure of LALF-14 bound to LPS as determined by the etNOESY experiment; the basic residues are drawn in blue, and the hydrophobic residues are shown in red. Only heavy atoms are displayed for clarity.

Molecular Modeling. Docking. In molecular docking calculations five 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 lowest final docked energy showed the same binding mode (Figure 2A). The common LPS binding motif (Table 2 of ref 5) proposes that the residue pairs Arg40/Arg41 and Lys47/ Lys64 correspond to Lys306/Lys351 and Lys439/Arg 382 of FhuA that bind to the phosphate groups of the LPS glucosamines GlcN II and GlcN I, respectively (Table 1). In the best docking model of the LALF-14 peptide (Figure 2A) the corresponding residue pairs are Arg40/Arg41 and Lys43/ Lys45, in slight disagreement with the proposed binding motif; the

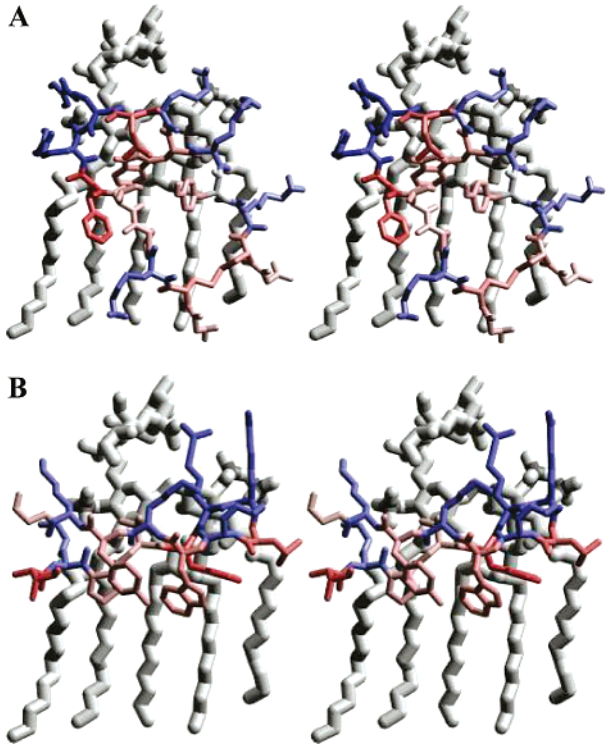

Figure 2. Structure of the LALF-14−LPS (A) and the $LALF_{34-49}$−LPS (B) complex calculated with AutoDock;[25] the LPS structure is from ref 4. The basic residues are drawn blue, and the hydrophobic residues are shown in red. Only heavy atoms are displayed for clarity.

discrepancy, however, is not unusual since residue Lys64 of the full-size protein is not present in the synthetic peptide fragment.

In another computer simulation the difference in conformations of the LALF-14 peptide when bound to LPS, as determined with etNOE, and the same fragment in the full-size protein was addressed. The LALF fragment 34−49 was cut out from the full-size protein and subjected to the same docking protocol as LALF-14. The resulting complex of $LALF_{34-49}$ with LPS (Figure 2.B) does not conform to the predicted LPS binding motif; the basic residue pairs of the peptide that bind to the LPS glucosamines GlcN II and GlcN I turn out to be Lys36/Lys47 and Arg40/Arg41, respectively.

Discussion

The etNOESY method to determine the three-dimensional structure of peptides bound to proteins, or other macromolecular systems, is becoming increasingly important in drug design efforts.[16] Reasonably accurate peptide structures can be determined with relatively few NOE interactions when the interactions occur between nonneighboring residues.[17]

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 transferred NOE effect are not always attainable. The etNOE is measured in conditions of approximately 10:1 peptide:LPS molar ratio, so experimental information is obtained neither on receptor (i.e. LPS) structure nor on 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. Additional knowledge is required in order to narrow the set of possible solutions.

Such additional knowledge can be derived from general binding motifs. In the case of LPS binding peptides we used knowledge from the proposed LPS-binding motif of proteins.[5] Among the proteins identified using the motif were many proteins that are known to bind LPS specifically (e.g. LALF) and that mediate the LPS-induced immune response.

In LALF, the proposed LPS-binding motif comprises Arg41, Arg40, Lys47, and Lys64; three of them are located in the amphipathic hairpin loop (residues 32−50) that was put forward as the LPS binding region[6] and are also part of LALF-14 and $LALF_{34-49}$. Docking calculations of the peptide fragment $LALF_{34-49}$, however, most strongly propose that Arg40 and Arg41 interact with lipid A GlcN I-phosphoryl, and Lys36 and Lys47 with GlcN II-phosphoryl, in disagreement with the binding motif that proposes Arg40 and Arg41 interacting with GlcN II phosphoryl and Lys47 with GlcN I-phosphoryl (Table 1). In fact, docking calculations of LPS with the full LALF[13] propose the same docking pattern as with $LALF_{34-49}$. A closer inspection of the complex shows that Lys47 and Lys64 in the full-size LALF protein are separated by the side-chain of Thr63 and can hardly interact with the same phosphoryl of LPS. This result indicates that not only does the spatial RMS superimposition of suitable basic side-chain pairs have to be taken into account when searching for the LPS binding motif[5] but also the full surface of the protein.

The docking calculations on LALF-14 indicate that its complex with LPS is different from the one of $LALF_{34-49}$. LALF-14 is a cyclic peptide with the disulfide bridge in a position that should stabilize the peptide backbone in a β-sheetlike conformation. The NMR

Table 1. Peptide Residues That Interact with Lipid A Phosphoryl Groups Attached to Glucosamine Residues I and II at Positions 1 and 4, Respectively

protein	GlcN I−1-phosphoryl	GlcN II−4-phosphoryl	source
FhuA	Arg382, Lys439	Lys306, Lys351	X-ray[4]
LALF	Lys47, Arg64	Arg40, Arg41	LPS binding motif[5]
$LALF_{34-49}$	Arg40, Arg41	Lys36, Lys47	docking calculation
LALF-14	Lys43, Lys45	Arg40, Arg41	docking calculation

results, however, show that the peptide has no regular secondary structure in water solution at 25 °C and that the latter is not formed upon interaction with LPS either. Other peptides have been reported that do not adopt the same conformation when free in solution and when being part of a larger protein chain.[7] The LPS-bound conformation of LALF-14 is still highly amphipathic and binds to LPS with an electrostatic (enthalpic) as well as a hydrophobic (entropic) component, the latter involving Pro37, Phe39, and Trp44.

In the absence of experimental data the modeled complexes of LPS and the peptides presented in this work have to be interpreted with caution. Recent approaches to isotopic labeling of peptides may open avenues to more direct studies of their complexes.[22]

The derived models are actively being used in structure-based antiseptic drug design providing leads to substances that may be clinically useful in the treatment of Gram-negative bacterial sepsis and shock.

Experimental Section

NMR. LALF-14 (GCKPTFRRLKWKYKCG) was synthesized at the Keck Biotechnology Resource Laboratory, Yale University, New Haven, CT (purity >99%). The NMR spectra were obtained in 90% H_2O−10% D_2O solution at 298 K and peptide concentrations from 1 to 2.5 mM. 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 10 ms and 70 ms) and two-dimensional NOE experiments (NOESY and ROESY, mixing times 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 exchange-transferred NOE (etNOE) experiments with mixing times of 80−200 ms were carried out using mixtures of LALF-14 and LPS that correspond to 11:1 w/w ratios of both components. A NOESY with a mixing time of 120 ms was used for structure determination.

Computational Methods. Three-dimensional structures of LALF-14 were computed using the torsion angle dynamics program DYANA.[23] The Cys48 β-β' cross-peaks were used for distance calibration. One hundred different structures were calculated; 10 structures selected according to lowest target function were energy minimized including distance restraints using the DISCOVER program (Accelrys, San Diego, CA) with the Consistent Valence Force Field (cvff[24]) on a Silicon Graphics O2 workstation; five structures with lowest target function were used for docking. The side-chains were kept uncharged during minimization in order to avoid charge repulsive effects; a distance-dependent dielectric constant was employed in order to simulate, at least in part, electrostatic screening by the solvent.

The coordinates of the lipid A portion of LPS from ref 4 (including two KDO residues) and the bound conformations of LALF-14 obtained 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 etNOE experiments while all side-chains (except that of Pro37) 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 deg, and a torsion step (tstep) of 5.0 deg, producing 20 structures for each starting structure. In the analysis step, 10 of the generated docked structures were evaluated. A similar protocol was employed for fragment $LALF_{34-49}$ from the full-size LALF protein.

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Appendix

Abbreviations. BPI, bactericidal/permeability increasing protein; GlcN, glucosamine; etNOE, exchange-transferred nuclear Overhauser effect; KDO, 3-deoxy-D-manno-2-octulosonate; LALF, limulus anti-LPS factor; LPS, lipopolysaccharide; NOESY, nuclear Overhauser effect spectroscopy; rmds, root-mean-square deviation; TNF-α, tumor necrosis factor α.

Supporting Information Available: Table of resonance assignments, chemical structure of a typical lipid A from *E. coli*, aromatic−aliphatic part of the exchange-transferred NOESY spectrum of LALF-14 in a mixture with LPS, and the coordinates of the LALF-14 ensemble. This material is available free of charge via the Internet at http://pubs.acs.org.

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