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Bioactive peptides derived from the *Limulus* anti-lipopolysaccharide factor: structure-activity relationships and formation of mixed peptide/lipid complexes

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Abstract: The design of peptides that would interact and neutralise bacterial endotoxins or LPS could have benefited from the analysis of comparative structure–activity relationships among close-related analogues. Here, we present a comparative structural characterisation of selected peptides derived from the LALF obtained by single-amino-acid replacement, which differ in biological activity. The peptides were characterised in solution using nuclear magnetic resonance, circular dichroism and fluorescence spectroscopies. Membrane mimetic peptide interactions were studied using fluorescence resonance energy transfer with the aid of extrinsic fluorescent probes that allowed the identification of mixed peptide/lipid complexes. Copyright © 2008 European Peptide Society and John Wiley & Sons, Ltd.

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Keywords: bioactive peptides; endotoxin; molecular recognition; LALF; lipid A; LPS; sepsis; septic shock

INTRODUCTION

LPS or bacterial endotoxin is the principal component of the gram-negative bacterial cell wall [1]. The basic LPS molecular structure consists of two distinct regions: a hydrophilic carbohydrate portion and outer core region, and the hydrophobic toxic lipid A component. This latter component is highly conserved among gram-negative bacteria and it has been reported that contributes to the toxicity of LPS [2]. Continuous exposure to LPS in mammalian bloodstream, either because of infection of gram-negative bacteria or as a consequence of intensive anti-microbial therapy, induces the deregulation of the release of inflammatory cytokines leading to a pathological condition known as septic shock characterised by hypotension, coagulopathy, organ failure and often death [1]. Circulating LPS in the blood is recognised and bound to LBPs. Among others, the LBP binds to LPS and the complex LBP/LPS interacts with a receptor, CD14, at the surface of the cells [3]. Then, the complex LPB/LPS/CD14 activates toll-like receptors (TLR2 and TLR4) that participate in the transduction of the LPS signal to the cell-nucleus that initiate the transcription of cytokine genes [3,4]. Efforts have been directed towards both, the characterisation of all the members and the full understanding of the LPS-signalling pathway in order to define pharmacological targets. However, although inhibitors of TNF-α factor and of other inflammatory mediators have been targeted for inhibition, so far this approach has not increased the survival of septic shock patients [5]. Therefore, increased interest has been credited to the inhibition of early events of the process. In particular, a therapeutic agent that can bind to and neutralise LPS directly has been proposed as a potential useful tool as a first stage towards the development of an effective, still to be discovered, LPS-neutralising drug [6–11]. Thus, it would be of interest to study the analysis of structure–activity relationships of synthetic LPS-binding peptides derived from well characterised natural LBPs. From this family of proteins, the LALF, from the horseshoe crab *Limulus polyphemus*, and the human BPI are the most well characterised. LALF is a small basic protein that binds to and neutralises the effect of LPS in the coagulation cascade of the crab [12]. BPI binds to LPS and could participate in the homeostasis of LPS-induced response [13,14]. The structures of both, LALF and BPI, have been solved by X-ray crystallography

Abbreviations: BPI, bactericidal/permeability-increasing protein; DPLA, 1-4′-diphosphoryl lipid A; DPPC, 1,2-dipalmitoyl-sn-glycero-3-phosphocholine; LALF, *Limulus* anti-LPS factor; LAL, *Limulus* amoebocyte lysate; LPS, lipopolysaccharide; LBP, lipopolysaccharide binding protein; PBS, phosphate buffered saline; SUV, small unilamellar vesicles.

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and structural analysis has revealed putative LPS-binding sites located at solvent exposed amphipathic β-hairpin structures [12,15,16]. Synthetic peptides derived from the LPS-binding domain of LALF have been reported as LPS-neutralising agents [17]; however, the structural information from these peptides, necessary for structure–activity relationship, is still limited. Two common features that have emerged for most LPS-neutralising peptides, derived or not from LPS-binding proteins, are a net positive charge and a putative tendency to adopt an amphipathic structure.

In this study, therefore, we described the structure in solution as determined by NMR of a synthetic LPS-binding cyclic peptide derived from LALF (LALF-14). The cyclic peptide adopted a nascent amphipathic β-hairpin-like structure with a non-canonical turn. In order to obtain structure–activity relationships on the role of such a turn, we have synthesized a full set of D-proline insertion and 'alanine scanning' analogues, analysed their solution structure and lipid interactions, and evaluated their biological activity.

MATERIAL AND METHODS

Peptide Synthesis and Purification

Peptides were manually synthesised by multiple solid-phase peptide synthesis using T-bag methodology [18] in Polystyrene Aminomethyl RAM (Rapp Polymer) resin that after final cleavage (70% TFA, 20% DCM, 5% H_2O, 2.5% TIBS and 2.5% EDT) gives *C*-terminal amidated peptides. Peptides were purified by preparative RP-HPLC to purities above 95%. Peptides containing Cys were cyclised by incubation of a 0.5 mg/ml peptide solution in a buffer containing 1.5% acetic acid, 0.9 M ammonium carbonate pH 7 and 15% DMSO for 24 h at room temperature [19]. The cyclic peptides were then purified by preparative RP-HPLC. Laser desorption MALDI-TOF was used to determine the identity of the peptides.

Nuclear Magnetic Resonance Spectroscopy (NMR)

All NMR experiments were performed on a Bruker DRX500 spectrometer on peptide samples of $\approx$1–2 mM concentration in pure D_2O and 90% H_2O/10% D_2O solutions at pH 5. All chemical shifts were internally referenced to the sodium salt of trimethylsilylpropionate (TSP). Phase-sensitive total correlation spectroscopy (mixing time 50 min) and nuclear Overhauser effect (NOE) spectroscopy (mixing time 150, 200 and 250 min) experiments were performed at 283 K, collecting 2048 points in f2 and 512 points in f1. Solvent suppression was achieved by selective pre-saturation during the relaxation delay (1.2 s) or field-gradient pulses. The proton resonances were assigned by the sequential assignment procedure [20]. Ranking of β-sheet population from CαH conformational chemical shifts have been calculated using the linear LALF-14 peptide as reference for the chemical shifts of the unfolded state.

Evaluation of LPS- and Lipid A-neutralising Activities of Peptides

All solutions used in the LPS-neutralising activity assays were tested to be endotoxin-free and the material was sterilised by heating for 3 h at 180 °C. Endotoxin-free water was from BioWhittaker and LPS from *E. coli* 0111:B4 and *E. coli* 055:B5 were from BioWhittaker and Sigma, respectively. LAL reagent contains a clottable protein that activates in the presence of non-neutralised LPS, and then the enzyme catalyses the splitting of *p*-nitroaniline (pNA) from the colourless chromogenic substrate Ac-Ile-Glu-Ala-Arg-pNA. Peptides were incubated with LPS (0.036 mg/ml) for 45 min at 37 °C. Addition of LAL was made to start the reaction. After 6 min at 37 °C, non-neutralised LPS was detected by incubation with chromogenic substrate for 10 min. Acetic acid (25% v/v final concentration) was added to stop the reaction and the pNA released was measured photometrically at 405 nm in a Rosys Anthos 2010 microtiter plate reader. Each experiment was done in triplicate and repeated in three independent microplates. The LPS-binding activities of the peptides were expressed as IC_{50} values obtained from serial dilution experiments. Similar procedures were used for the evaluation of the peptides as neutralising agents of the endotoxic component lipid A. In the later experiments DPLA – Sigma was used replacing LPS.

Fluorescence Spectroscopy Studies

All the fluorescence studies were made in an LS-50B Perkin Elmer fluorescence spectrometer using PBS solution. To analyse the effect of the peptides on the critical micellar concentration (cmc) of DPLA and SDS the fluorescence emission of the probe 1,3-diphenyl-1,3,5-hexatriene (DPH – Sigma) was used.

Preparation of Lipid Vesicles

The DPPC was purchased from Sigma. Small unilamellar vesicles (SUV, DPPC : LPS, 8:2, mol : mol and DPPC : DPLA, 8:2) were prepared in PBS pH 7.2 by sonication of phospholipid dispersions as described earlier [21].

Circular Dichroism Experiments

CD spectra were recorded with a Jasco-810 spectropolarimeter. Unless otherwise stated, the buffer for CD assays was 5 mM MOPS-NaOH, pH 7.0 in quartz cells of 0.1 cm path. The CD spectra were the average of five to seven scans, made at 0.2 nm intervals, and always the same buffer and/or lipid solutions without peptides, used as baseline, were subtracted.

Inhibition of LPS-Induced TNF-α Release in Mice

A concentration of 10 µg of LPS (*Salmonella minesotta*), pre-mixed with 100 µg of peptides, or 10 µg of polymyxin B in 100 µl of PBS, was injected intra-peritoneally into BALB/c mice (7/group). A concentration of 10 µg of LPS in 100 µl of PBS injected intra-peritoneally was used as positive control. Animals were bled after 60 min and blood was stored for subsequent TNF-α assays at −80 °C. The TNF-α ELISA was performed according to the manufacturer's instructions (Genzyme) using 50 µl serum dilution (1/5) samples.

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Table 1 Biological activity of D-Pro insertion and 'Ala-scanning' analogues of LALF-14

Name	Sequence[a]	LPS-neutralising activity[b] (IC_{50}, μM)	DPLA-neutralising activity[b] (IC_{50}, μM)
LALF-14	G(CK^{36}PTFRRLKWKYK^{47}C)G	50	40
LALF-40	G(CKPTF**p**RRLKWKYKC)G	>250	—
LALF-41	G(CKPTFR**p**RLKWKYKC)G	>250	—
LALF-42	G(CKPTFRR**p**LKWKYKC)G	>250	—
LALF-43	G(CKPTFRRL**p**KWKYKC)G	80	106
LALF-44	G(CKPTFRRLK**p**WKYKC)G	50	48
LALF-F39A	G(CKPT**A**RRLKWKYKC)G	60	—
LALF-R40A	G(CKPTF**A**RLKWKYKC)G	50	—
LALF-R41A	G(CKPTFR**A**LKWKYKC)G	30	—
LALF-L42A	G(CKPTFRR**A**KWKYKC)G	20	—
LALF-K43A	G(CKPTFRRL**A**WKYKC)G	50	—
LALF-W44A	G(CKPTFRRLK**A**KYKC)G	150	—
LALF-K45A	G(CKPTFRRLKW**A**YKC)G	55	—
LALF-Y46A	G(CKPTFRRLKWK**A**KC)G	200	—
LALF-K47A	G(CKPTFRRLKWKY**A**C)G	50	—

[a] Lower case letter represents D-amino acids. Sequence in parenthesis indicates that the peptide is cyclised by means of an S–S bound between the Cys residues.
[b] IC_{50} = concentration necessary to neutralise 50% of LPS (or DPLA) as determined by a serial dilution assay. Data expressed as mean (n > 3). The Standard Deviation of the data was always lower than 10%.

RESULTS AND DISCUSSION

LALF-14 was developed from the LPS-binding domain of the LBP (i.e. LALF) [17]. The peptide was shown to bind *in vitro* to LPS and to decrease the levels of TNF-α in an *in vivo* septic shock murine model [17]. LALF-14 (Table 1) is a cyclic 16*mer* peptide derived from a solvent exposed hairpin in the structure of LALF (residues 36–47 with two pairs Gly–Cys and Cys–Gly at the *N*- and *C*-terminus, respectively); however, only recently, the actual structure of the peptide in solution and when bound to the LPS has been analysed by Pristovsek *et al.* [22]. These authors suggested that the peptide lacks regular secondary structure neither in aqueous solution nor in the presence of LPS. Nevertheless, our comparative study using LALF-14 and several derivative peptides suggests that the presence of a 'nascent' secondary structure in the peptide LALF-14 could be related to its biological activity.

Solution Structure of LALF-14

The initial structural characterisation of LALF-14 and all LALF-14 substitution analogues in the present study was performed by CD spectroscopy. The CD spectra in PBS buffer of LALF-14 and most of the analogues analysed (Figure 1(A)) were defined by a positive ellipticity band around 230 nm and a strong negative ellipticity band around 200 nm that were slightly different between the different analogues. These spectra are characteristic of peptides that adopt mixed conformations populating unordered and extended β-sheet structures [23].

The NMR spectra of peptides having a well-defined secondary structure are characterised by a large spread of chemical shifts, while unordered structures show much less dispersion due to the structural variability of peptides that, in turn, leads to averaged resonance of frequencies. Complete assignments of the chemical shifts were then made for LALF-14 and for a non-cyclised analogue, linear analogue (LALF-14l), using COSY, TOCSY and ROESY spectra as described [24]. $^1H_\alpha$ conformational shifts (difference between the observed $^1H_\alpha$ chemical shifts of the studied peptide and those from a random-coil reference peptide) are good indicators of secondary structure formation [25]. In particular, a β-hairpin conformation is characterised by negative and positive conformational shifts of the αH in the turn and the strands, respectively. The non-cyclised analogue of LALF-14 (LALF-14l) is unstructured. Therefore, LALF-14l was used as random-coil reference peptide to estimate conformational chemical shifts for the peptide series presented in this work. This procedure will ensure the accuracy of the data because it has been reported that short, unfolded peptides traditionally used as references, can adopt non-random conformations [26] and that aromatic or other residues can contribute to the $^1H_\alpha$ proton chemical shifts of neighbour residues [27]. It is known that cyclation through a disulfide bond between Cys residues located at the termini of a peptide whose sequence already has some propensity to fold

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into a β-hairpin remarkably increases the population of the β-conformer [17,28–30]. As expected, the cyclic peptide LALF-14 with a disulfide bridge was more structured, and populated a β-hairpin conformation with the right strand register (Figure 1(B)). Long- and medium-range contacts detected by NMR confirmed the formation of the amphipathic β-hairpin (Figure 1(C)). A hydrophobic cluster is formed at one of the faces by residues Pro^{37}, which faces Tyr^{46} (pair Pro^{37}–Tyr^{46} or Pro^{4}–Tyr^{13} following the LALF protein or LALF14c peptide sequence numbering, respectively), and Phe^{39} which faces Trp^{44} (pair Phe^{39}–Trp^{44}). A cationic face is formed by residue pairs Lys^{36}–Lys^{47} and Thr^{38}–Lys^{45}.

A previous NMR-based conformational study of LALF-14 suggested that the peptide at 298 K adopted predominantly highly flexible structures [22]. However, we performed our study at 283 K in order to decrease temperature factors that could preclude the identification of nascent-like structures. These kinds of structures can emerge in the unfolded conformation of a peptide and pre-organise all subsequent folding events that will drive towards the bioactive conformation.

D-Pro Insertion and Ala-scanning Analogues of LALF-14

Gellman and co-workers [31] have synthesised a variety of β-sheet hairpins containing type II′ (or I′) turns stabilised by a D-amino acid (D-Pro) and Gly in the $i+1$ and $i+2$ positions of the loop. We wanted to know whether the stabilising effect of D-Pro in different types of β-turns could be also applicable to LALF-14 and how different turn geometries could influence the biological activity. To this end, we synthesised a set of D-Pro insertion analogues of LALF-14 and the biological activity was evaluated. We determined both the LPS- and DPLA-neutralising activities (Table 1). Insertion of D-Pro at the *N*-end of the β-turn has a more deleterious effect on biological activity than when it is inserted close to the *C*-end. In fact, peptides LALF-43 and -44 with D-Pro inserted after Leu^{42} and Lys^{43}, respectively, retained the biological activity of the original peptide LALF-14 while peptides LALF-40, -41 and -42 with D-Pro insertions after Phe^{39}, Arg^{40}, Arg^{41}, respectively, showed a reduced LPS-neutralising activity (Table 1). In an attempt to analyse the structure–activity relationship, all four D-Pro insertion analogues were analysed by CD and NMR. The analysis of both, the conformational shifts (Figure 2) and NOEs (Fig. S.1 in Supplementary Information), suggested that the insertion of D-Pro all along the sequence of the β-turn of the original LALF-14 peptide induced a position-dependent partial destabilisation of such a turn. The insertion of D-Pro before the turn (LALF-40), namely, between Phe^{39} and Arg^{40} abruptly disrupted the β–hairpin conformation by destroying the stabilizing interactions at the hydrophobic patch. In fact, only long-range NOEs could be detected close

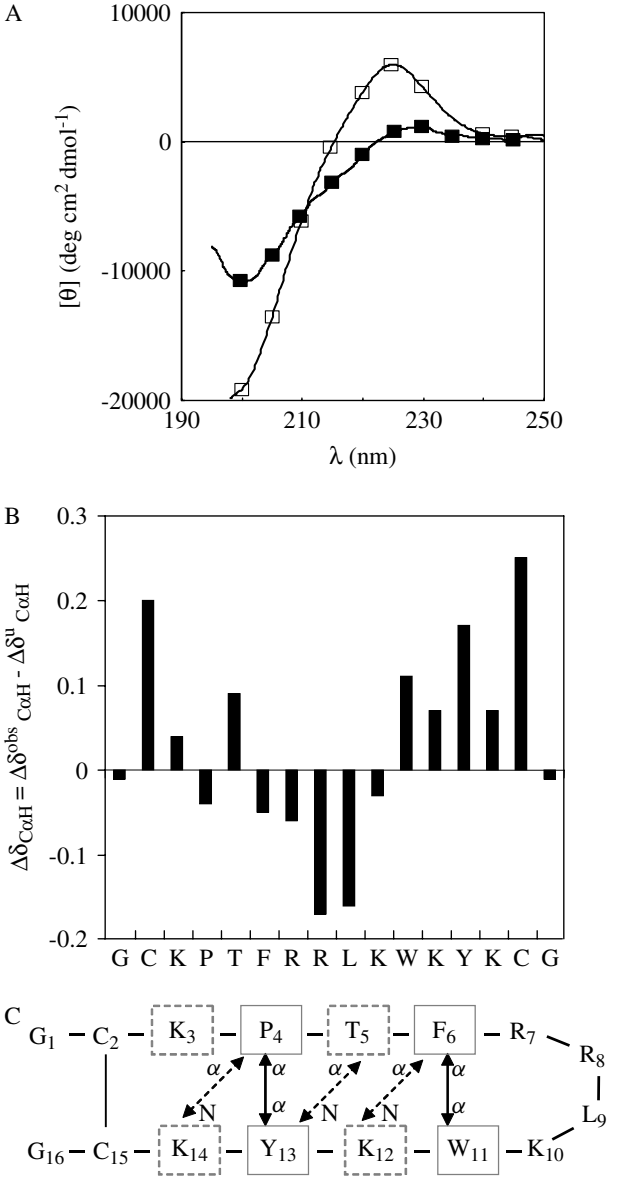

Figure 1 (A) Far-UV CD spectra of LALF-14 and LALF-44 (empty and filled squares, respectively) at 50 μM concentration in 5 mM MOPS-NaOH buffer, pH 7.0. (B) Observed conformational CαH chemical shift increments ($\Delta\delta_{C\alpha H}$) for LALF-14 relative to the chemical shifts of the unfolded peptide LALF-14l. (C) Schematic representation of the amphipathic β–hairpin formed by the cyclic peptide LALF-14. Pairs Pro^{4}–Tyr^{13} and Phe^{6}–Trp^{11} form a hydrophobic patch and Lys^{3}–Lys^{14} and Thr^{5}–Lys^{12} a cationic face. Continuous lines indicate covalent bonds and arrows long-range contacts detected by NMR (continuous arrows Hα–Hα NOEs and discontinuous arrows Hα-NH NOEs).

to the disulfide bond, between residues Lys^{36} and Tyr^{46}. The insertion of a D-Pro residue within the turn sequence, between Arg^{40} and Arg^{41} (LALF-41) was not as disrupting as in the former case, but it changed the strand register impairing the hydrophobic interactions at the hydrophobic face. According to the long-range NOEs observed, Lys^{36} interacts with Tyr^{46} and Thr^{38}

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with Trp[44]. As a result, LALF-41 is less folded than the original LALF-14. D-Pro between Arg[41] and Leu[42] (LALF-42) again changed the strand register and led to a less folded peptide than LALF-41. For LALF-42, only the contacts between Lys[36]–Tyr[46] seemed to be strengthened and interactions between Thr[38] and Trp[44] disappeared or were weaker. Peptide LALF-43 was more structured than the other three peptides with D-Pro insertions. Although the insertion of D-Pro between residues Leu[42] and Lys[43] impaired a proper strand arrangement, Hα–Hα NOEs confirmed that the interacting pair Pro[37]–Tyr[46] was recovered, but no NOEs supporting the pair Phe[39]–Trp[44] were found. In this case, Trp[44] and Lys[45] displayed some NMR contacts to Arg[40] and Arg[41]. In the peptide LALF-44 (CD spectrum is shown in Figure 1(A)) the insertion of the D-Pro residue was performed after the original turn, i.e. between residues Lys[43] and Trp[44]. According to our NMR data, Lys[43]–D-Pro formed a two-residue turn that led to a much distorted β–hairpin where Tyr[46] and Trp[44] interact with Thr[38] and Leu[42]. The β–hairpin content ranking of these peptides according to the number of long and medium relevant NMR contacts is as follows: LALF-14 > LALF-43 > LALF-44 > LALF-41 ≈ LALF-42 ≫ LALF-40. The biological activity (Table 1) measured is LALF-14 = LALF-44 > LALF-43 > LALF-41 ≈ LALF-42 ≈ LALF-40. Therefore, only those peptides where a nascent β-hairpin-like structure with two faces of different polarity could be stabilised displayed remarkable LPS-neutralising activity.

We next investigated the relative importance of each amino acid from Phe[39] to Lys[47] by using an L-Ala-scanning mutagenesis approach. The alanine analogues were subjected to determination of the LPS-neutralising activity (Table 1) and initial structural characterisation by CD. As mentioned earlier, the CD spectra were similar to that of the original peptide (Figure 1(A)). However, the LPS-neutralising activity was different among the alanine analogues. Replacement of the aromatic residues Trp[44] and Tyr[46] (peptides LALF-W44A and LALF-Y46A) induced a severe decrease in activity while alanine substitution at other positions showed to be almost neutral. These results demonstrate that the aromatic residues Trp[44] and Tyr[46] constitute key residues for LALF-14 activity and suggest that these residues contribute to the stabilisation of the hydrophobic face of the putative hairpin that was shown to be of importance for activity. In this line of evidence, a recent study on designed LPS-neutralising peptides, highlighted the importance of the hydrophobic amino acids as key elements to bind to the acyl chains of the LPS molecule [32].

Interaction of LALF-14 and LALF-44 with Model Lipid Membranes

An LPS is released from bacterial cell wall in an inactive micellar form that dissociates to the active

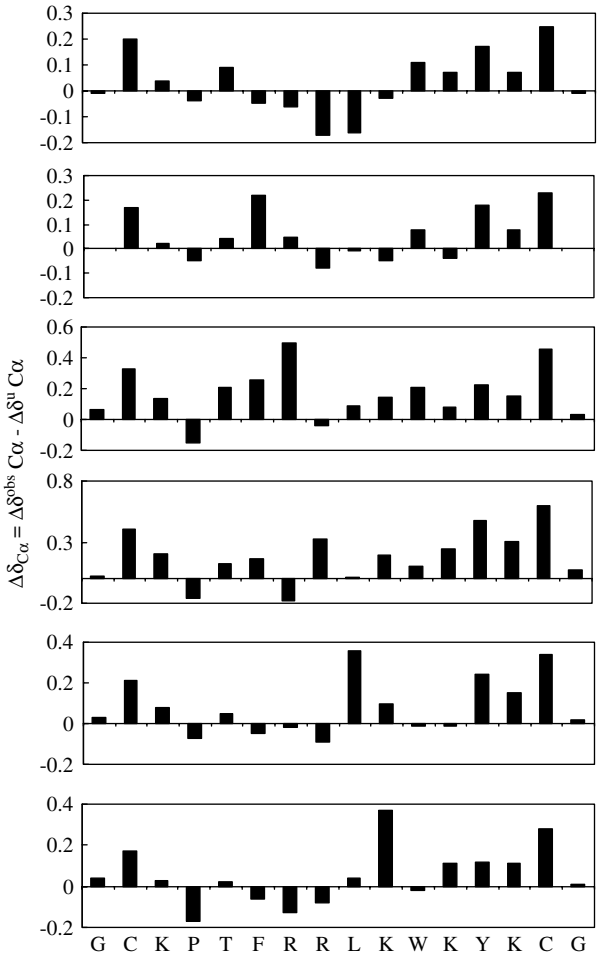

Figure 2 Observed conformational CαH chemical shift increments ($\Delta\delta_{C\alpha H}$) for D-Pro insertion analogues of LALF-14 relative to the chemical shifts of the unfolded peptide LALF-14l. From top to bottom, LALF-14, LALF-40, LALF-41, LALF-42, LALF-43 and LALF-44.

monomeric form at slow rate [33] and LPS-binding proteins as LBP could catalyse the process [3,34–36]. It has been reported that different families of cationic antimicrobial peptides that block LPS-induced macrophage activation, can bind to LPS and inhibit the binding of LPS to LBP, thus preventing the LBP-mediated transfer of LPS to CD14 [37–39]. Furthermore, it has been also hypothesised that the complex LBP-monomeric LPS could serve as an alternative detoxification way involving the participation of additional lipid binding proteins [40,41]. These data suggest that LPS-neutralising peptides should interact and induce a re-organisation of the micellar LPS. Thus, we next analysed the binding to different lipid models (i.e. lipid vesicles and micelles) of two representative active peptides, namely, LALF-14 and -44.

The NMR data suggested that the solution structure of LALF-44 was less well defined than the β-hairpin structure obtained for LALF-14. However, the two peptides have similar biological activity as molecules

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with capability to *in vitro* neutralise both LPS and its endotoxic lipid A component (Table 1). These results suggest that the two peptides could be induced into a bioactive conformation upon binding to the target lipids. We initially analysed the binding of LALF-14 and -44 to vesicles containing negatively charged lipids (small unilamellar vesicles – SUV-DPPC : LPS, (8 : 2 mol : mol) and SUV-DPPC : lipid A, (8 : 2 mol : mol)). As mentioned earlier, the CD spectra of LALF-14 and -44 in aqueous buffer (Figure 1(A)) were representative of a mixture of conformations. However, in the presence of SUV-DPPC : LPS (Figure 3(A)), the spectra were characterised by the presence of a strong negative ellipticity band centred at 205 nm that has been attributed to the stabilisation of extended β-sheet structures [42]. In contrast, a more canonical β-sheet (negative ellipticity band close to 215 nm and positive ellipticity band below 200 nm) spectra were obtained for both peptides in the presence of SUV-DPPC : lipid A (Figure 3(B)). These results suggested that peptides LALF-14 and -44 bind to LPS and lipid A when these two lipids are present in membranes. However, in contrast to other amphipathic cationic peptides that binding to biological membranes was associated to an antibiotic activity [43,44], LALF-14 and -44 did not show antibiotic activity at peptide concentrations up to 50 μM. In addition, these peptides are not able to form transmembrane channels when analysed by the method of pH gradient using lipid vesicles with an entrapped pH-sensitive dye (not shown) [45].

Next, we wanted to explore whether or not these peptides perturb the micellar organisation of LPS or lipid A. The equilibrium between monomer and aggregated forms in micellar systems could be evaluated by the analysis of the cmc with the aid of the fluorescent probe 1,6-diphenyl-1,3,5-hexatriene (DPH). Incorporation of DPH into the core of micelles is accompanied by the strong enhancement of its fluorescence [46,47]. Titration of a 5 μM DPH solution in PBS buffer (λexc = 380 nm; λem = 430 nm) with different concentrations of lipid A in the presence or absence of LALF-14 and -44 suggested that the peptides decreased the cmc of lipid A from 1 to 0.3 μM (Fig. S.2 in Supplementary Information). The same experimental conditions were applied to LPS; however, the extremely low value of cmc [33] precluded an accurate analysis. These results suggested that the peptides stabilised the micellar form of lipid A. We evaluated the putative formation of mixed lipid/peptide micelles in a model system where the micellar perturbation induced by the peptides could be analysed in a wider range of lipid concentrations. The SDS is an anionic surfactant that has been used as membrane mimetic due to its intrinsic properties [48,49]. SDS exists as monomer in solution or in a micellar state at concentrations above the cmc and thus it is useful to analyse the formation of lipid/peptide mixed complexes. In fact, we obtained a cmc of 4 mM

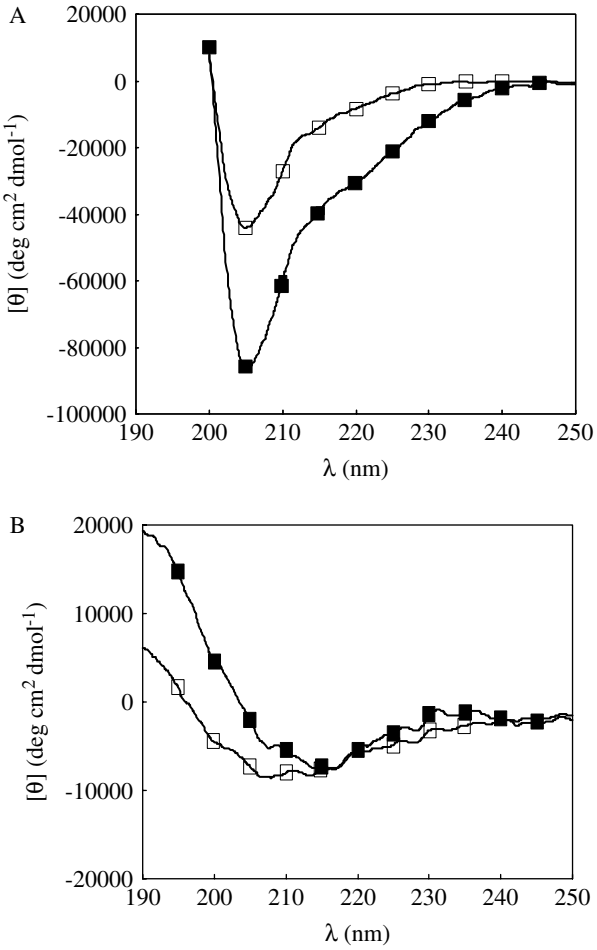

Figure 3 Far-UV CD spectra of LALF-14 and LALF-44 (empty and filled squares, respectively) at 30 μM in the presence of: (A) SUV-DPPC : LPS (8 : 2, 3 mM) and (B) SUV-DPPC : lipid A (8 : 2, 3 mM).

for SDS in PBS buffer (Figure 4(B)); however, in the presence of peptides, the fluorescence profile was difficult to analyse suggesting the presence of putative peptide/SDS micellar complexes. The emission maximum of the aromatic residues of LALF-14 averaged at 345 nm (in plain buffered solution the emission maximum was 356 nm) in the presence of concentrations of SDS above 0.1 mM. Furthermore, when LALF-14 and -44 peptides, in the presence of 5 μM DPH were excited at 280 nm, only the fluorescence emission of Trp and Tyr residues is recovered with emission maxima at 354 nm (Figure 4(A)). However, when the peptide LALF-14 and the probe were excited at 280 nm in the presence of increasing concentrations of SDS, two emission maxima were observed (Figure 4(A)) corresponding to the Trp/Tyr fluorescence at short wavelengths (emission maxima around 340 nm) and the probe fluorescence at longer wavelengths (emission maxima centred at 430 nm). Then the Trp/Tyr residues of LALF-14 should be close to the DPH probe and they contributed to its fluorescence because of energy transfer. However,

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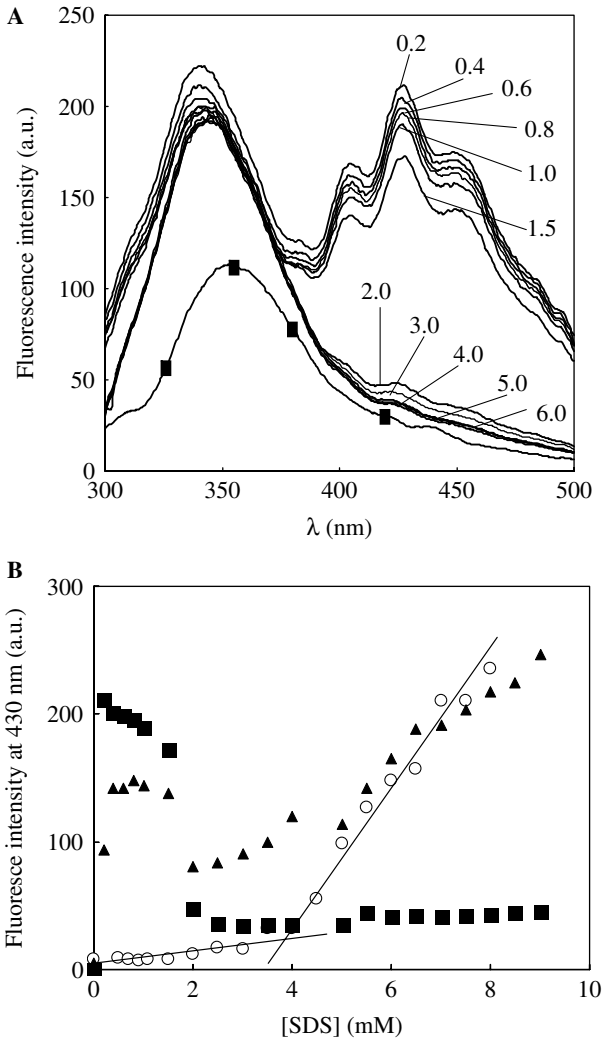

Figure 4 Fluorescence spectroscopy analysis of the formation of peptide/SDS mixed micelles. (A) Fluorescence emission spectra obtained by excitation at 280 nm of a mixture of DPH (5 μM) and LALF-14 in the absence (line with black squares) and in the presence of SDS at concentrations (in mM) indicated. (B) Fluorescence emission intensity of the probe DPH at 430 nm upon excitation at 380 nm in the absence (open circles) or in the presence of LALF-14 at 50 μM (triangles). The black squares are the values of the fluorescence intensity obtained from the DPH probe at 430 nm in the presence of LALF-14 at 50 μM upon excitation of the aromatic residues of the peptide at 280 nm.

when the SDS concentration was increased from 2 up to 10 mM, close to or above the cmc, respectively, the energy transfer was not observed. Figure 4(B) shows the fluorescence intensity obtained at 430 nm (from the DPH) in different conditions. Initially, the analysis of such fluorescence intensity at different SDS concentrations was used upon direct excitation of the probe (λexc = 340 nm) for the determination of the cmc of SDS (open circles in Figure 4(B)). When the same analysis was performed in the presence of LALF-14 (triangles in Figure 4(B)), we obtained an increase of the fluorescence intensity at SDS concentrations below 2 mM, then a decrease, and at SDS concentrations above 4 mM the fluorescence intensity was similar to that obtained in the absence of LALF-14. However, when we excited the aromatic amino acids of LALF-14 (λexc = 280 nm), we only detected DPH emission at SDS concentrations below 2 mM (black squares in Figure 4(B)). The same experimental procedure was evaluated using LALF-44 with identical results. The data provide support to the formation of a specific peptide-surfactant complex at pre-micellar concentrations. Pre-micellar surfactant solutions (surfactant concentration below cmc) may be considered to exist in a distribution of surfactant aggregates, which are not fully formed micelles [50]. These results suggest that the monomeric peptide in solution binds to SDS monomers and the formation of a mixed peptide/SDS complex can be induced. The structure of the complex should be well defined because it allows the process of energy transfer to occur between the aromatic rings of the Trp and Tyr residues and the fluorescent probe.

LALF-14 and LALF-44 Inhibit LPS-Stimulated TNF-α Release in a Murine Endoxemia Model

Several studies have consistently reported elevated levels of TNF-α in a large number of septic shock patients experiencing early death or suffering of prolonged fatal multiple organ failure [51]. Using a murine endotoxic shock model [17], we assessed the effects of peptides LALF-14 and -44 on LPS activity *in vivo*. Administration of these compounds markedly decreased the peak serum levels of TNF-α (Figure 5). In this assay, we found that 100 μg of LALF-14 or LALF-44 showed the same activity that 10 μg of the reference compound polymyxin B as inhibitors of LPS-induced TNF-α release.

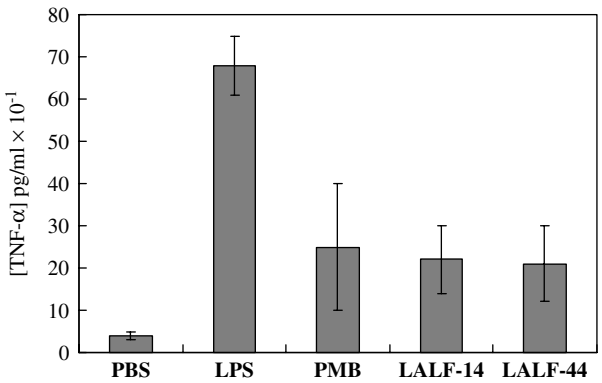

Figure 5 Inhibition of LPS-induced TNF-α release in mice by LALF-14 and LALF-44. Data expressed as mean ± SD (n > 5).

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CONCLUDING REMARKS

In conclusion, our results suggest that LPS-neutralising peptides have a high tendency to form peptide/lipid mixed complexes. In addition, there is a correlation between LPS-neutralising activity and the ability to populate β-hairpin-like nascent secondary structures. Such properties could be of relevance for the biological activity of this class of LPS (or endotoxin)-neutralising peptides that, almost certainly, depends on a process of molecular recognition between the LPS and the peptide followed by an LPS-dependent induction of bioactive conformation on the peptide.

Supplementary Material

Supplementary electronic material for this paper is available in Wiley InterScience at: http://www.interscience.wiley.com/jpages/1075-2617/suppmat/

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