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The influence of rough lipopolysaccharide structure on molecular interactions with mammalian antimicrobial peptides

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ABSTRACT

The influence of *Escherichia coli* rough lipopolysaccharide chemotype on the membrane activity of the mammalian antimicrobial peptides (AMPs) human cathelicidin (LL37) and bovine lactoferricin (LFb) was studied on bilayers using solid state ^{2}H NMR (ssNMR) and on monolayers using the subphase injection technique, Brewster angle microscopy (BAM) and neutron reflectivity (NR). The two AMPs were selected because of their differing biological activities. Chain-deuterated dipalmitoylphosphatidylcholine (d_{62}-DPPC) was added to the LPS samples, to highlight alterations in the system properties caused by the presence of the different LPS chemotypes and upon AMP challenge. Both LPS chemotypes showed a temperature dependent influence on the packing of the DPPC molecules, with a fluidizing effect exerted below the DPPC phase transition temperature (T_m), and an ordering effect observed above the T_m. The magnitude of these effects was influenced by LPS structure; the shorter Rc LPS promoted more ordered lipid packing compared to the longer Ra LPS. These differential ordering effects in turn influenced the penetrative activity of the two peptides, as the perturbation induced by both AMPs to Ra LPS-containing models was greater than that observed in those containing Rc LPS. The NR data suggests that in addition to penetrating into the monolayers, both LL37 and LFb formed a non-interacting layer below the LPS/DPPC monolayer. The overall activity of LL37, which showed a deeper penetration into the model membranes, was more marked than that of LFb, which appeared to localise at the interfacial region, thus providing evidence for the molecular origins of their different biological activities.

1. Introduction

The external leaflet of the outer membrane (OM) of Gram-negative bacteria is mainly composed of the essential, negatively charged, macroamphiphile lipopolysaccharide (LPS). In bacteria under physiological conditions, OM LPS is cross-linked with Mg^{2+} ions and acts as the first line of defence against environmental perturbations, competing microorganisms and in the case of symbiotic bacteria, against the host's immune system [1–4]. The protective role of the OM affects also the microbicidal activity of drugs used in the treatment of bacterial infections [3,5] leading to a reduced susceptibility of Gram-negative bacteria to common antiseptics and antibiotics compared with Gram-positives [6,7]. By virtue of their ability to effectively breach the barrier of the OM, particular attention has been paid to antimicrobial peptides (AMPs) as possible therapeutics or adjuvant treatments for highly resistant microbial infections [8,9].

Different Gram-negative bacterial strains may express structurally diverse LPS chemotypes (Fig. 1) with distinct physico-chemical properties which impart different characteristics to the OM [10]. Mutant strains expressing truncated, so-called rough LPS chemotypes are more susceptible to antibiotics, when compared to the wild-type, and, because of this enhanced vulnerability they are considered to be suitable for peptide–membrane interaction studies designed to elucidate the mechanism of action of AMPs on the OM [4,11]. The membrane disrupting activity of AMPs depends on the active conformation adopted by the peptides as well as the composition of the membrane [12]. To date however, AMP–membrane interaction studies have largely ignored the effect of LPS on membrane models [13–16].

In this study we have investigated the influence of both peptide conformation and LPS chemotype on their molecular interactions, using the α-helical human peptide cathelicidin (LL37) and the β-sheet-forming bovine lactoferricin peptide (LFb) together with two LPS chemotypes from *Escherichia coli* rough mutants. The range of MIC values for LL37 [17,18] and LFb [19–21] can vary largely across literature depending on the *E. coli* strain and the test conditions used. The reported MIC values for LL37 and LFb against the smooth *E. coli* ATCC 25922 are

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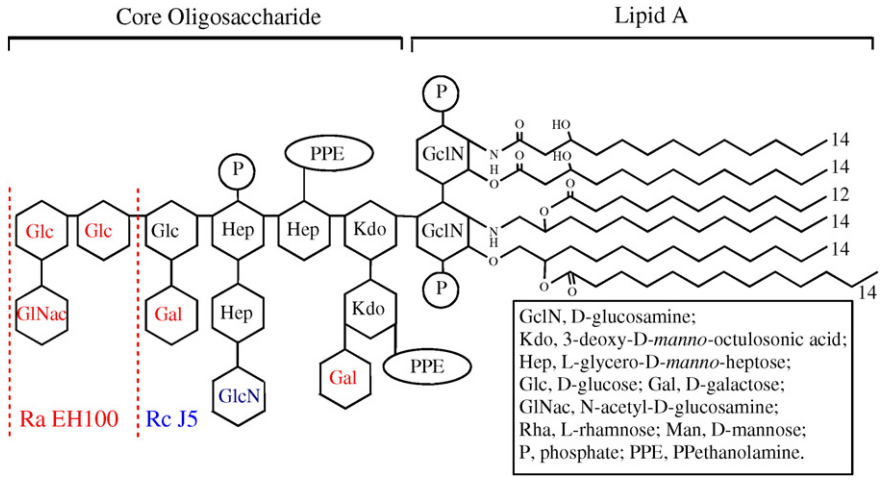

Fig. 1. Chemical structure of the rough LPS chemotypes Ra EH100 and Rc J5. Adapted from Inagaki *et al.* [38]. Sugars in red are not present in the Rc J5 LPS chemotype.

respectively ~0.6 μM [22] and 10 μM [23], suggesting that LL37 is ~16 times more active than LFb. With regard to their mechanisms of action, both peptides have been shown to interact with, perturb and eventually permeabilise both natural bacterial membranes and synthetic lipid membranes [20,24–29]. LPS has been shown to be a determinant for LL37 and LFb binding and activity [30–34], making an investigation into their molecular interactions with the OM a key step in understanding their different efficacies against *E. coli* (and possibly other Gram negatives). The realisation of such an investigation necessitates the use of suitable OM mimetics which will remain stable over the timescales needed to conduct biophysical experiments. For example, LPS has been successfully incorporated into planar artificial membrane mimetics stabilised with dipalmitoylphosphatidylcholine (DPPC), for use in neutron reflectivity studies [35]. For the interaction studies presented here, the OM mimetics used were monolayers and multilamellar liposomes composed of mixtures of *E. coli* EH100 Ra LPS or *E. coli* J5 Rc LPS (Fig. 1), together with chain-deuterated and fully hydrogenated DPPC. The DPPC provides a platform into which to anchor the LPS chains without imposing any additional packing constraints on planar membrane mimetics, due to its cylindrical molecular shape. The zwitterionic nature of DPPC reduces the likelihood of any direct electrostatic interaction with AMPs, allowing us to focus on the interactions promoted by the LPS alone.

The order parameters of the fatty acyl moieties of the chain-deuterated d_{62}-DPPC within multilamellar liposomes were used in this study to examine the influence of LPS on bilayer order in solid state ^{2}H NMR (ssNMR) experiments [36]. Liposomes containing mixtures of h-DPPC and d_{62}-DPPC with either J5 Rc LPS or EH100 Ra LPS were analysed by ssNMR in order to examine the effects of the two LPS chemotypes on bilayer packing, thus aiding the understanding of the structural role of LPS in the OM of bacteria. The same technique was also used to study the effect of the interaction of the two AMPs on the DPPC acyl chain order parameters of the liposomes in the presence of 20 mol% LPS [37].

Mixed monolayers of rough LPS chemotypes and d_{62}-DPPC were studied in neutron reflectivity (NR) and Brewster angle microscopy (BAM) experiments in order to characterise their behaviour at the air/liquid interface [39]. Monolayers were also used to study the kinetics and magnitude of peptide interactions with each LPS chemotype following subphase injection, and in order to determine the length of time needed to allow equilibrium to be reached in the subsequent NR studies, following challenge by AMPs [13,40–42].

These biophysical observations on the role of LPSs in the membrane models were devised to elucidate the stabilising role of different rough LPS chemotypes on the OM structure as well as their influence on the activities of LL37 and LFb.

2. Experimental section

2.1. Materials

Rc LPS from *E. coli* J5 (impurities: protein 1.4%, nucleic acid 0.340%), was obtained from Merck KGaA (Darmstadt, Germany) and was used without further purification. LPS from *E. coli* EH100 was obtained from Sigma-Aldrich Ltd. (Dorset, U.K.) and used after purification following the method described elsewhere [30]. LL37 peptide was obtained from GenScript USA Inc. (Piscataway, USA) with a purity > 94%. The LFb peptide was obtained from Alpha Diagnostic International Inc. (San Antonio, USA) with a purity > 90%. Both peptides were used as supplied. Deuterium oxide D_2O 99.990 at.% D and $MgCl_2$ anhydrous were also supplied by Sigma Aldrich Ltd. The lipids, hydrogenated 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (h-DPPC) and chain deuterated 1,2-dipalmitoyl-d_{62}-sn-glycero-3-phosphocholine (d_{62}-DPPC) were obtained from Avanti Polar Lipids (Alabaster, USA) and used without further purification. Chloroform and methanol were obtained from Fisher Scientific (Loughborough, U.K.). Ethanol (99%) was obtained from VWR International (Lutterworth, U.K.). All the organic solvents used were of analytical grade or better. The ultrapure water at 18.2 MΩ cm was obtained from a Purelab Ultra machine from ELGA Process Water (Marlow, U.K.). Whatman chromatographic paper No. 1 from Merck KGaA (Darmstadt, Germany) was used to produce the Wilhelmy plates required for the monolayer surface pressure measurements.

2.2. Solid state ^{2}H NMR measurements

The phospholipid/LPS mixtures used to produce the multilamellar liposomes for the ^{2}H NMR experiments contained 30 or 40 mol% d_{62}-DPPC in lieu of the equivalent amount of h-DPPC. The pure DPPC lipid mixture contained 40 mol% d_{62}-DPPC with 60 mol% h-DPPC. The lipid mixtures Eh-20 and J5-20 contained 20 mol% of either Ra EH100 LPS or Rc J5 LPS respectively, 30 mol% of d_{62}-DPPC and 50 mol% h-DPPC, giving a total mass of 4 mg with 80 mol% phospholipid and 20 mol% LPS. A summary of the acronyms used to describe the lipid/LPS mixtures used in the experiment and their relative compositions is reported in Table 1. The composition of the vesicles in consistent with the composition of the monolayers used in the Langmuir monolayers and NR experiments.

Where necessary, at the mixing stage, the peptides were added to the lipid mixture solutions to give final lipid/peptide molar ratios of either 50:1 or 100:1, from solutions prepared in ethanol. The mixtures were bath-sonicated for 5 min at 22 °C temperature in an Elmasonic P ultrasonic bath (Elma Hans Schmidbauer GmbH & Co. KG, Singen,