Structure and Association of Human Lactoferrin Peptides with Escherichia coli Lipopolysaccharide

Daniel S. Chapple, Rohanah Hussain, Christopher L. Joannou, Robert E. W. Hancock, Edward Odell, Robert W. Evans, and Giuliano Siligardi

Metalloprotein Research Group, Randall Centre for Molecular Cell Biology, Guy's Campus, King's College London, London SE1 1UL, Drug Interaction Activity Screening Unit, Department of Pharmacy, King's College London, London SE1 8WA, and Department of Oral Medicine and Pathology, King's College London, Guys Hospital, London SE1 9RT, United Kingdom, and Department of Microbiology and Immunology, University of British Columbia, Vancouver, British Columbia V6T 1Z3, Canada

Received 30 June 2003/Returned for modification 25 November 2003/Accepted 10 February 2004

An 11-amino-acid amphipathic synthetic peptide homologous to a helical region on helix 1 of human lactoferrin HLP-2 exhibited bactericidal activity against Escherichia coli serotype O111, whereas an analogue synthesized with Pro substituted for Met, HLP-6, had greatly reduced antimicrobial activity. The bactericidal activity of HLP-2 was 10-fold greater than that of HLP-6 in both buffer and growth medium by time-kill assays. These assays also showed a pronounced lag phase that was both concentration and time dependent and that was far greater for HLP-2 than for HLP-6. Both peptides, however, were shown to be equally efficient in destabilizing the outer membrane when the hydrophobic probe 1-N-phenylnaphthylamine was used and to have the same lipopolysaccharide (LPS) binding affinity, as shown by polymyxin B displacement. Circular dichroism (CD) spectroscopy was used to study the structure and the organization of the peptides in solution and upon interaction with E. coli LPS. In the presence of LPS, HLP-2 and HLP-6 were found to bind and adopt a β-strand conformation rather than an α-helix, as shown by nonimmobilized ligand interaction assay-CD spectroscopy. Furthermore, this assay was used to show that there is a time-dependent association of peptide that results in an ordered formation of peptide aggregates. The rate of interpeptide association was far greater in HLP-2 LPS than in HLP-6 LPS, which was consistent with the lag phase observed on the killing curves. These results allow us to propose a mechanism by which HLP-2 folds and self-assembles at the outer membrane surface before exerting its activity.

Cationic antimicrobial peptides represent possible new chemotherapeutic agents based on naturally occurring proteins and peptides. To date, hundreds of such peptides have been isolated throughout nature from single-celled microorganisms, mammals, amphibians, birds, fish, and plants (10), indicating their importance in the innate immune system (2, 9, 11). The mechanisms by which these peptides kill microorganisms remain unclear, although the cationic charge and high proportion of hydrophobic amino acids common to most of these peptides are thought to play important roles. Apart from the cationic charge, the structures of cationic antimicrobial peptides are also thought to play a major role in antimicrobial activity. Although certain peptide structural groups have been noted, including amphipathic α helices, β structures, extended structures, and loops, no overall conservation of amino acids exists (3, 12). Furthermore, peptides can fold in such a way as to segregate the charged or hydrophilic portions from the hydrophobic residues, thus permitting these highly charged peptides to interact with bacterial membranes (16). Structural studies have determined that some antimicrobial peptides that do not have a disulfide bond tend to be irregular in solution, forming a structure upon binding to bacterial membranes (1, 7, 24).

While there has been some clarification of the mode of interaction of cationic antimicrobial peptides with model membranes, few studies have clarified the interaction with the bacterial outer membrane. The current view (23) of how peptides interact with the outer membrane has been adapted from studies of polycationic antibiotics, such as aminoglycosides and polymyxins. Bacterial outer membranes are unusual asymmetric membranes containing the polyanionic glycolipid lipopolysaccharide (LPS) in the outer leaflet and phospholipids in the inner leaflet (13). To stabilize the anionic surface of the outer membrane, the LPS is partially neutralized by divalent cations, such as Mg$^{2+}$ and Ca$^{2+}$. Cationic peptides can interact at these divalent cation binding sites on LPS, thereby distorting the integrity of the outer membrane. It has been demonstrated that many polycations bind with micromolar affinity to purified LPS both in the free form and when they are associated with cells. The disruption of the cell surface has been observed both as blebs arising from the cell surface and by measurement of the uptake of probes, like the hydrophobic probe 1-N-phenyl-naphthylamine (NPN), which is normally excluded by the outer membrane. However, other details of this interaction have not been clarified, including the mode of binding at the cell surface and whether binding to the LPS results in a structural transition in the peptide.
Previous work with a synthetic 11-amino-acid peptide derived from a surface region on human lactoferrin has demonstrated that the peptide causes membrane destabilization, followed by the collapse of membrane integrity and cell death (5). An analogue of HLP-2, named HLP-6, which has a Pro substitution for a Met, had greatly reduced activity compared to that of HLP-2. We had previously hypothesized that this difference is due to the structural changes caused by the Pro substitution, as the charge of the peptide remains the same (6). It has also been demonstrated that HLP-2 is capable of binding to LPS, making this membrane constituent a prime target for the initial bacterium-peptide interaction (5).

In this paper we describe a detailed study of the activities of HLP-2 and HLP-6 against *Escherichia coli* serotype O111. The actions of the peptides on the outer membrane were investigated, and the structures that HLP-2 and HLP-6 adopt upon interaction with *E. coli* LPS were determined by nonimmobilized ligand interaction assay-circular dichroism (CD) spectroscopy (NILIA-CD). By comparing the differences between HLP-2 and the HLP-6 analogue, we have gained insight into the mechanism of initial interaction with *E. coli* cells. We are able to propose a hypothesis whereby there is a structurally dependent ordered association of the peptide at the outer membrane as a first stage in antibacterial activity.

**MATERIALS AND METHODS**

Peptide synthesis. Peptides HLP-2 and HLP-6 were synthesized by using 9-fluorenylmethoxycarbonyl chemistry at the University of British Columbia, Vancouver, British Columbia, Canada. The peptides were assessed to be >95% pure by reverse-phase high-pressure liquid chromatography and mass spectrometry. The peptide HLP-2 sequence is NH2-FQWQRNMRKVR-COOH, and HLP-6 had a Pro substitution for a Met in HLP-2 (NH2-FQWQRNPRKR-COOH). Bacterial strain. *E. coli* NCTC 8007 serotype O111 was obtained from the National Collection of Type Cultures, London, United Kingdom. Antibacterial activities of peptides. Time-kill assays were performed with *E. coli* in both suspension medium (phosphate-buffered saline [PBS]) and complex growth medium (1% proteose peptone) by standard microbiological techniques. Cells from an overnight culture were inoculated into fresh medium and grown to an initial bacterium-peptide interaction (5).

**Peptide binding to *E. coli* LPS.** The interaction of the peptides with *E. coli* LPS (Highly Purified; Sigma) was demonstrated by competition binding with dansyl polymyxin (DPX) by previously described methods (8). The amount of DPX leading to saturation of LPS was determined by adding DPX in increments of 0.5 μM to 2 μg of LPS per ml in 5 mM HEPES (pH 7.2) buffer and measuring the increase in fluorescence. Inhibition of DPX binding was carried out by adding 1 ml of LPS at a concentration of 3 μg/ml in 5 mM HEPES (pH 7.2) to a cuvette and adding DPX to give 90% saturation of LPS binding. Aliquots (10 μl) of the peptide solution were added to the cuvette, and fluorescence was monitored until maximal inhibition was reached. The maximal inhibition was determined to be the concentration of peptide that produced no further decrease in fluorescence. The experiment was repeated with polymyxin B as a positive control, buffer only as a negative control, and human lactoferrin (Sigma).

**CD.** The CD spectra were obtained by using nitrogen-flushed JASCO J-720 and J-810 spectropolarimeters. The measurements were carried out in a 1-cm cell for the near-UV region (230 to 340 nm) and a 0.05-cm cell for the far-UV region (190 to 250 nm). Sodium dodecyl sulfate (SDS) was added to the peptides in both the submicelle and the micelle forms. Peptides were also added to 100% 2,2,2-trifluoroethanol (TFE) to look for the helix-forming properties of the peptides.

**LPS interactions.** A 25-μg/ml stock solution of LPS in water was made, and a 10-μl aliquot was added to 180 μl of peptide solution (0.2 mg/ml in water). The structure was measured by using the spectropolarimeters described above, and a further 10 μl of LPS solution was added. This was repeated at least three times to give the CD spectra of the peptides in the presence of increasing concentrations of LPS. A time course study was undertaken, in which the CD spectra of the peptides in the presence of LPS were measured continuously for 5 h and then again after 24 h. Controls were undertaken with LPS in water alone, and light-scatter artifacts were determined by comparing the results for samples of LPS in the presence and the absence of peptides.

**Molecular modeling.** Molecular modeling of peptides was performed on the basis of the global energy minimization of peptide structures by using Insight II software (Molecular Simulation, Cambridge, United Kingdom) on a Silicon Graphics workstation.

**RESULTS**

**Activities of HLP-2 and HLP-6 against *E. coli***. Time-kill studies carried out with *E. coli* and peptides HLP-2 and HLP-6 in PBS or growth medium (1% proteose peptone) and by monitoring of viable cell counts clearly showed that HLP-2 was far more active than HLP-6 (Fig. 1 and Fig. 2A to C). Moreover, there was less than a twofold difference between the minimally effective concentration and the concentration of HLP-2 giving 100% killing (Fig. 1). By assessment of the time course of killing, a clear lag time was observed for HLP-2 (Fig. 2B), whereas the lag time was far less pronounced for HLP-6 (Fig. 2B). The lag time was proportional to the peptide concentration, as illustrated when a time course study with fixed peptide concentrations (Fig. 2A), chosen from the suboptimal concentrations detected in Fig. 1, was carried out. This suggests that a two-stage process in the antibacterial action of HLP-2 was taking place. HLP-6, however, gave rise to a linear killing curve, suggesting that its antibacterial activity is directly proportional to the peptide concentration (Fig. 2B).

**Killing curve studies carried out in complex growth medium** showed that HLP-2 concentrations above 250 μM were bactericidal, resulting in rapid cell death (Fig. 2C). In the case of HLP-6, the peptide was bacteriostatic; and at sublethal concentrations, the cells eventually recovered to reach maximal cell counts after 18 h (Fig. 2C).

**Permeabilization of the outer membrane of *E. coli*** by HLP-2 and HLP-6. The destabilization of the outer membrane by peptides was assessed by using the fluorescent probe NPN (19). The probe, which is nonpolar and weakly fluorescent in aqueous solution, is normally excluded by the outer membrane.
When the outer membrane surface is disrupted, NPN can partition into the hydrophobic core of the outer membrane of bacteria, where it fluoresces strongly. Therefore, NPN can be used to determine the actions of peptides on outer membrane stability. Both peptide HLP-2 and its analog, HLP-6, interacted with the outer membrane of \textit{E. coli}, causing an increase in NPN fluorescence which was proportional to the peptide concentration (Fig. 3A). This suggests that both peptides have similar abilities to permeabilize the outer membrane of \textit{E. coli} to NPN, even though HLP-2 had a far greater bactericidal activity.

**Affinities of HLP-2 and HLP-6 binding to \textit{E. coli} LPS.** The fluorescence of DPX is greatly enhanced upon binding to the divalent cation binding sites of LPS and can be used to determine the relative LPS-binding affinities of peptides on the basis of their abilities to competitively displace DPX from LPS (20). Peptides HLP-2 and HLP-6 equally displaced DPX, whereas no displacement was seen for human lactoferrin at the concentrations tested (Fig. 3B). Thus, both peptides, which carry charges of 3+, would seem to have approximately the same affinity for LPS. Therefore, the ability of HLP-2 and HLP-6 to bind to LPS and permeabilize the \textit{E. coli} outer membrane does not reflect antimicrobial activity.

**CD analysis of peptide structure.** Although HLP-2 corresponds to an \(\alpha\)-helical region in the intact lactoferrin molecule, an irregular structure by CD spectroscopy was observed for the free peptide in aqueous solution, indicating the lack of an ordered structure. The same result was observed for HLP-6. The addition of SDS to the peptide at a final concentration of 1.35 mM, to mimic the submicelle form, also showed spectra characteristic of irregular structures for both HLP peptides; and a very small amount of ordered structure was seen upon the addition of 20 mM SDS, which mimicked the micelle form. The replacement of water by TFE, which creates a more hydrophobic environment and which promotes the ability of peptides to adopt \(\alpha\)-helical structures, still resulted in spectra typical of irregular structures for both HLP-2 and HLP-6. Only upon the addition of 30 mM NaOH, which neutralized the positive charge of the peptide, was HLP-2 seen to adopt a significant content of helical conformation in TFE solution, but this was not observed with HLP-6 (data not shown).

**NILIA-CD of HLP-2 and HLP-6 with LPS.** Although neither HLP-2 nor HLP-6 adopted an ordered conformation in aqueous solution, the CD spectra revealed that a structural change had taken place in the presence of \textit{E. coli} LPS (Fig. 4A and B, respectively). The experiments were carried out with molar excesses of the HLPs due to the problem of light scattering caused by the formation of micelles and vesicles in the presence of high concentrations of LPS. Subtraction of the CD spectra of the HLPs from those of the HLP-LPS complex showed a significant \(\beta\)-strand contribution for the LPS-bound peptides. The tendency for HLP-2 to form a \(\beta\) strand was observed to be greater than that for HLP-6.

**Time-dependent conformational changes of HLP-2 and HLP-6 upon interaction with LPS.** Scanning of the aromatic region (230 to 320 nm) showed that the changes occurring within the LPS complexes of HLP-2 and HLP-6 were both time dependent (Fig. 5A and B) and temperature dependent (data not shown). However, when these changes were plotted as a function of time at 235 nm, the rate of change by HLP-2 was far greater than that by HLP-6; the CD spectrum for HLP-2 reached a plateau after 2 h, whereas that for HLP-6 was still rising after 5 h. At 2 h, the intensity of the CD spectrum that relates to the peptide association upon interaction with LPS was twice as great for HLP-2 as for HLP-6 (Fig. 6). An increase in the temperature from 25 to 37°C increased the rates at which both peptides associated with the LPS, although this had a more pronounced effect on HLP-2 (Fig. 6).

**DISCUSSION**

HLP-2, a synthetic peptide derived from a region on helix 1 of human lactoferrin, is thought to play a major role in deter-
FIG. 2. Antibacterial effects of HLP-2 and HLP-6 on *E. coli* at timed intervals in suspension medium and growth medium. The residual viable *E. coli* cells were monitored at time intervals as the numbers of CFU per milliliter and compared to those for control cells (■). (A) Effects of sublethal concentrations of HLP-2 over time. Peptide was added to washed cells corresponding to 10⁸ cells ml⁻¹ in PBS to a final concentration of 129 (○), 258 (▲), or 323 (▲) μM; and the effects on viability were determined at timed intervals. (B) Effects of lethal and sublethal concentrations of HLP-6 over time. Peptide was added to washed cells in PBS to a final concentration of 330 (○), 660 (▲), or 990 (▲) μM; and the effects on viability were determined at timed intervals. (C) Effects of lethal and sublethal concentrations of HLP-2 and HLP-6 on actively growing cells. Peptides were added to cultures in culture medium (1% proteose peptone), with cell counts corresponding to 5 × 10⁵ cells ml⁻¹, to a final concentration of 125 (○) or 250 (▲) μM for HLP-2 or 660 (▲) or 990 (▲) μM for HLP-6; and the effects on viability were monitored over time. Each datum is the mean of at least three experiments, and each experiment was carried out in triplicate. Error bars are plotted as standard errors of the means.
mining the antimicrobial activities of lactoferrin and its peptide degradation products. It has been predicted to form an amphipathic α helix (5, 6, 21), similar to its disposition in the native molecule lactoferrin. In this study it has been revealed by CD spectroscopy that HLP-2 exists as an irregular structured peptide in solution, and only upon binding to bacterial LPS does it form a structure. However, the dominating structure was of a β-strand-type conformation rather than the α-helical conformation. The less potent HLP-6 analogue, which was synthesized with Pro substituted for Met, was designed to disrupt the potential α-helical structure by introducing a kink (25) and also formed a β-strand-type structure in association with LPS. When HLP-2 was modeled as a β-type structure, there was still an amphipathic face of charged residues (data not shown). Previous studies have emphasized the folding of various peptides into defined secondary structures after they associate with membranes (1, 7) and have concluded that this manifests itself at the level of the cytoplasmic membrane, whereby the free energy gained in folding drives the insertion of the peptide into the cytoplasmic membrane (14). Thus, when it is considered how peptides interact with and kill bacteria, it is important to consider whether contact with the first membrane accessed by the peptide, namely, the outer membrane, will induce folding of the peptide into its final membrane-associated form. Our

![Graph A](image1)

**FIG. 3.** (A) Permeabilization of the *E. coli* outer membrane by HLP-2 and HLP-6 in the presence of NPN. Peptides were sequentially added to cells, and changes in the fluorescence intensity of the NPN probe were monitored. ●, HLP-2; ▼, HLP-6; ■, control with peptide and NPN. (B) Displacement of DPX from *E. coli* LPS by HLP-2, HLP-6, and lactoferrin. Peptides were sequentially added to *E. coli* LPS in the presence of DPX, and changes in the fluorescence intensity of the probe were monitored. Results are expressed as the percentage of fluorescence compared to the fluorescence for LPS-DPX complexes alone. ●, HLP-2; ▼, HLP-6; ■, lactoferrin. Each datum is the mean ± standard deviation of three experiments, and in all cases the standard deviation was less than 10%. Error bars were therefore excluded for clarity.
studies indicate that this is indeed so, and the implications are that the folded structure is important for the interaction with and/or passage across the outer membrane. However, even though the two peptides were almost equally effective at binding to LPS and at inducing nonspecific outer membrane permeabilization, this did not equate with bactericidal activity. In the case of HLP-6, *E. coli* cells in growth medium with HLP-6 were able to recover, suggesting that other events are required for a cationic peptide to be highly effective and that simply coating the bacterial surface is not adequate.

The time lag observed for HLP-2 when detailed killing curve studies were carried out suggested that a two-stage process that was both concentration and time dependent was taking place. The lag may be a prerequisite for the substantially enhanced antibacterial activity exhibited by HLP-2, because HLP-6, which had a far lower level of activity against *E. coli*, did not exhibit this property and must therefore be acting in a different manner. NILIA-CD revealed that HLP-2 in the presence of *E. coli* LPS was initiating an interaction that was time dependent and that correlated with the observed lag (Fig. 1B). Examina-
tion of the CD spectra for the aromatic regions indicated that in the presence of LPS, HLP-2 was self-associating in an ordered manner, possibly forming a tightly packed aggregate or array of peptides. Such a raft of peptides would have a very powerful ability to disrupt the outer membrane structure. Individual peptide molecules in these aggregates might be arranged to give both hydrophobic and hydrophilic faces, resulting in a very substantive disruption of the polyanionic surface of the outer membrane. The concerted interaction of a polycationic surface with the outer membrane would disrupt the fluidity of the bacterial membrane, permitting the translocation of peptide molecules across the outer membrane and access to other lethal targets. Indeed, the slow rate of formation of these peptide rafts is consistent with the observed lag times in the various actions of the peptides, if one considers that the presentation of peptide to the lethal target is dependent on the gradual buildup of these rafts. The CD spectra measured at a single wavelength as a function of time showed saturation types of profiles for both peptides (Fig. 6). However, the plateau for HLP-6 was reached after a longer time than

FIG. 5. Changes in CD spectra of HLP-2 (A) and HLP-6 (B) in the presence of LPS over time. The peptide concentration was 130 μM, and the ratio of the LPS concentration to peptide concentration was 1:468.
that for HLP-2. The proline substitution in HLP-2 therefore affected the capability of the peptide to pack and form these aggregates at the rate observed for HLP-2.

These conclusions are consistent with the modified “self-promoted uptake” model proposed by Hancock and Chapple (10), although the original model did not consider that the aggregation of polycations contributed to crossing of the outer membrane. They are, however, consistent with the observation that many peptides form multiple blebs at the surface of the outer membrane as part of their actions against cells (5, 22). This impressive expansion of the outer membrane is consistent with the insertion of peptide rafts into the outer membrane. We propose that many other peptides have a similar mechanism of interaction with the outer membrane, although the kinetics of aggregate formation may vary substantially with more potent peptides. The results for HLP-2 presented in this paper indicate that this mechanism needs to be modified, in that the peptides themselves are proposed to act as a point of nucleation for the formation of dense plaques.

Lactoferrin has evolved to be susceptible to digestion by host proteases which generate potent peptides that contain the HLP-2 region at sites where high concentrations of lactoferrin will be present, for instance, in the infant gut and at sites of inflammation (4). Peptides generated from bovine lactoferrin have been demonstrated to be present in mouse models (18), and this can be extrapolated to the humans. Lactoferricin B, the HLP-2 equivalent in bovine lactoferrin, has also been shown to adopt a β-strand conformation in the presence of lipid (15). Structure-function studies with an equivalent peptide derived from bovine lactoferrin demonstrated its interaction with bacterial phospholipid membranes (17). It seems possible, therefore, that both bovine and human lactoferrin-derived peptides have similar mechanisms of bactericidal activity.

ACKNOWLEDGMENTS

We thank ULIRS for the use of the facilities. Susan Farmer, UBC, is thanked for technical support and advice.

We thank the Canadian Bacterial Diseases Network for funding to R.E.W.H., who received salary support from a Medical Research Council of Canada Distinguished Scientist Award. We thank the Special Trustees of St. Thomas’ Hospital and Numico Research (Wageningen, The Netherlands) for financial support.

REFERENCES


