Structure-Function Relationship of Antibacterial Synthetic Peptides Homologous to a Helical Surface Region on Human Lactoferrin against Escherichia coli Serotype O111

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Lactoferrin is an avidly iron-binding glycoprotein of the transferrin family. It is found at mucosal surfaces, within the specific granules of neutrophils (32), and in biological fluids (31), such as milk (6, 7, 22), from which it was first isolated. Lactoferrin has been shown to have antimicrobial activity against a broad range of gram-positive bacteria, gram-negative bacteria, and fungi (3, 5, 24). This activity was attributed to its ability to sequester two iron atoms (35), an essential bacterial nutrient, and is restricted to the apoprotein, with the dimeric form being inactive (3). Lactoferrin has also been shown to act synergistically with other proteins, such as lysozyme (13) and immunoglobulin A (1), suggesting that it may damage the cell wall or outer membrane. Furthermore, lactoferrin binds to lipid A (2) and porins (15) and induces lipopolysaccharide (LPS) release from the bacterial wall (14).

Proteolytic digestion of human lactoferrin in vitro yields a peptide fragment called lactoferricin H, which has enhanced antimicrobial activity (4). The peptide corresponds partially to the amino-terminal lobe of lactoferrin. Synthetic peptides homologous to this region and analogs have been shown to have activity against Staphylococcus aureus and an Acinetobacter strain. The mode of action of human lactoferrin peptide (HLP) 2 against E. coli serotype O111 (NCTC 8007) was established by using flow cytometry, surface plasmon resonance, and transmission electron microscopy. Flow cytometry was used to monitor membrane potential, membrane integrity, and metabolic processes by using the fluorescent probes bis-1,3-(dibutylbarbituric acid)-trimethine oxonol, propidium iodide, and carbonyl cyanide m-chlorophenylhydrazone, respectively. HLP 2 was found to act at the cell membrane, causing complete loss of membrane potential after 10 min and of membrane integrity within 30 min, with irreversible damage to the cell as shown by rapid loss of viability. The number of particles, measured by light scatter on the flow cytometer, dropped significantly, showing that bacterial lysis resulted. The peptide was shown to bind to E. coli O111 lipopolysaccharide by using surface plasmon resonance. Transmission electron microscopy revealed bacterial distortion, with the outer membrane becoming detached from the inner cytoplasmic membrane. We conclude that HLP 2 causes membrane disruption of the outer membrane, resulting in lysis, and that structural considerations are important for antibacterial activity.

Lactoferricin includes an 11-amino-acid amphipathic alpha-helical region which is exhibited on the outer surface of the amino-terminal lobe of lactoferrin. Synthetic peptides homologous to this region exhibited potent antibacterial activity against a selected range of both gram-negative and gram-positive bacteria. An analog synthesized with methionine substituted for proline at position 26, which is predicted to disrupt the helical region, abolished antibacterial activity against Escherichia coli and considerably reduced antibacterial activity against Staphylococcus aureus and an Acinetobacter strain. The mode of action of human lactoferrin peptide (HLP) 2 against E. coli serotype O111 (NCTC 8007) was established by using flow cytometry, surface plasmon resonance, and transmission electron microscopy. Flow cytometry was used to monitor membrane potential, membrane integrity, and metabolic processes by using the fluorescent probes bis-1,3-(dibutylbarbituric acid)-trimethine oxonol, propidium iodide, and carbonyl cyanide m-chlorophenylhydrazone, respectively. HLP 2 was found to act at the cell membrane, causing complete loss of membrane potential after 10 min and of membrane integrity within 30 min, with irreversible damage to the cell as shown by rapid loss of viability. The number of particles, measured by light scatter on the flow cytometer, dropped significantly, showing that bacterial lysis resulted. The peptide was shown to bind to E. coli O111 lipopolysaccharide by using surface plasmon resonance. Transmission electron microscopy revealed bacterial distortion, with the outer membrane becoming detached from the inner cytoplasmic membrane. We conclude that HLP 2 causes membrane disruption of the outer membrane, resulting in lysis, and that structural considerations are important for antibacterial activity.

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selected range of gram-negative and gram-positive microorganisms were assessed. By using *E. coli*, flow cytometry was used to monitor changes in bacterial cell wall potential (29, 30) and cell membrane integrity (17), with specific fluorescent dyes, transmission electron microscopy (TEM) to detect morphological changes of bacterial cells, and surface plasmon resonance to detect specific peptide binding sites. The implications of these findings with respect to the antimicrobial activity of full-length lactoferrin in vivo are discussed.

**MATERIALS AND METHODS**

Peptide synthesis. Peptides HLP 1, 6, and 7 were synthesized by using 9-fluorenylmethoxycarbonyl chemistry at Kings College Pharmacy Department, London, United Kingdom. Peptides were assessed to be >95% pure by reverse-phase high-pressure liquid chromatography and mass spectrometry. Peptide HLP 2 was synthesized by Neosystem Laboratoire, Strasbourg, France. Peptide HLP 1 corresponded to the loop region of human lactoferricin (residues 20 to 35; NH₂-FQWQRKMRKVRGPRPV-SOOH), HLP 2 corresponded to the alpha-helical region of the loop in its native conformation (residues 20 to 30; NH₂-FOWQRKNRMKVGRPV-SOOH), HLP 6 had a prolinate substituted for methionine in HLP 2 (NH₂-FOWQRKMRKVRGPRPV-SOOH), and HLP 7 was the d form of HLP 2.

*Bacterial strains.* Staphylococcus aureus NCTC 10571 and *E. coli* NCTC 8007 and NCTC 10418 were obtained from the National Collection of Type Cultures, Colindale, United Kingdom. Classical isolates of an *Acinetobacter sp.*, *Enterobacter aerogenes*, a *Klebsiella sp.*, *Providencia stuartii*, and *Proteus mirabilis* were provided by the Department of Microbiology, St. Thomas's Hospital, London, United Kingdom. Bacterial cultures were stored at −70°C and grown on Columbia blood agar at 37°C.

**MIC determination.** MICs of each peptide were determined in 96-well plates. Plates were dissolved in 1% proteose peptone and serially diluted in microtiter wells to give concentrations of between 1.3 mM and 10 μM in a final volume of 95 μl. Bacteria were incubated at 37°C overnight in 1% proteose peptone to give approximately 10⁸ bacteria/ml, and 5 μl was added to each well. The plates were incubated at 37°C overnight, and growth was determined by absorbance at 620 nm. Antimicrobial activity was expressed as the concentration of the peptide required to give no increase in absorbance at 620 nm following incubation (MIC). The MIC determination for each peptide was repeated in separate plates at least four times.

**Effects of HLP 2 on membrane integrity and potential.** The membrane potential and integrity of *E. coli* NCTC 8007 in the presence of HLP 2 was monitored by using flow cytometry and selected dyes by the method of Mason et al. (30). *E. coli* incubated overnight in 1% proteose peptone and serially diluted in microtiter wells to give concentrations of between 1.3 mM and 10 μM in a final volume of 95 μl. Bacteria were incubated at 37°C overnight in 1% proteose peptone to give approximately 10⁸ bacteria/ml, and 5 μl was added to each well. The plates were incubated at 37°C overnight, and growth was determined by absorbance at 620 nm. Antimicrobial activity was expressed as the concentration of the peptide required to give no increase in absorbance at 620 nm following incubation (MIC). The MIC determination for each peptide was repeated in separate plates at least four times.

**Effect of HLP 2 on bacterial lysis and cell morphology.** Lysis and cell morphology of the bacteria were assessed. By using *E. coli*, flow cytometry was used to monitor changes in bacterial cell wall potential (29, 30) and cell membrane integrity (17), with specific fluorescent dyes, transmission electron microscopy (TEM) to detect morphological changes of bacterial cells, and surface plasmon resonance to detect specific peptide binding sites. The implications of these findings with respect to the antimicrobial activity of full-length lactoferrin in vivo are discussed.

**RESULTS**

**Antibacterial activities of HLPs.** HLP 1, the loop region, was active against *E. coli* NCTC 8007 and *S. aureus* NCTC 10571 with the same potency and had greater activity against an *Acinetobacter* strain but had no activity against *P. mirabilis* (Table 1). HLP 2, the alpha-helical region, had greater activity than HLP 1 against *E. coli* NCTC 8007, *S. aureus*, and *P. mirabilis* and showed activity against *P. mirabilis* and *S. aureus* NCTC 10571 and *S. aureus*. HLP 5, in which methionine 26 was replaced by proline, showed no activity, up to 1 mM, against either *E. coli* NCTC 8007 or *P. mirabilis* and reduced activity (compared with HLP 2) against *S. aureus* and the *Acinetobacter* strain. The d form of HLP 2 (HLP 7) showed increased activity against *E. coli* NCTC 8007 and the *Acinetobacter* strain and the same potency against *S. aureus* as HLP 2 but had no activity against *P. mirabilis*.

**Effects of HLP 2 on the membrane potential and integrity.** The number of cells in the bacterial population which showed dye-associated fluorescence in the presence of DiBAC₄(3) or PI was expressed as a percentage. The dye-response assay (Fig. 1a) demonstrated that the addition of HLP 2 to *E. coli* NCTC 8007 resulted in 50% fluorescence at approximately 100 μM (0.15 μg/ml), and in the presence of PI at approximately 140 μM (0.2 μg/ml), showing that the collapse of membrane potential and integrity occurred at approximately the same concentrations. Time-response assays (Fig. 1b) showed that 50% fluorescence is seen after approximately 8 min in the presence of DiBAC₄(3) and after approximately 35 min in the...
presence of PI. Collapse of membrane potential therefore occurred before collapse of membrane integrity.

**Metabolic activity.** Inhibition of the metabolic pathways by addition of the respiratory poison CCCP or of enzymatic pathways by reduction of the assay temperature to 4°C had no effect on the activity of HLP 2 (Table 2).

**Rate of cell lysis.** The results were plotted as a percentage of particles compared to that for a control culture which contained no peptide. The dose-response assay (Fig. 2a) showed that 50% of the particles remained at approximately 140 μM (0.2 mg/ml), and the time-response assay (Fig. 2b) showed that 50% of particles remained after 50 min. A comparison of the time-response assays and dose-response assays for particle number with the corresponding DiBAC₄(3) and PI assays indicated an inversely proportional relationship between PI fluorescence (indicative of membrane integrity) and particle number.

**HLP 2 binding to LPS.** After taking into account any non-specific binding by subtracting binding to a blank chip surface, 450 ± 50 resonance units of HLP 2 bound to 787 ± 100 resonance units of LPS, which is indicative of a specific interaction between HLP 2 and LPS.

**Effects of HLP 2 on permeabilization of E. coli.** Treatment of E. coli with peptide HLP 2 at the MIC for 2 h resulted in a shift of both forward and side light scatter (Fig. 3), indicating a change in size or morphology (29).

**Electron microscopy studies of E. coli cells treated with HLP 2.** Upon exposure of E. coli to HLP 2 at the MIC and half the MIC for 2 h, TEM revealed clumping of the cytoplasm and the presence of ghost cells (cells which have no cytoplasm but still have a cell wall) (Fig. 4b) as compared to control cells (Fig. 4a). At half the MIC, cytoplasmic clumping and enlargement of the bacteria, caused by blistering of the outer membrane, were observed (Fig. 4c, d, and e). The outer membrane was observed to separate, particularly at the ends of the cells or at the junction of dividing cells. No changes were seen at concentrations lower than half the MIC or after 1 h at the MIC or half the MIC. The change in the morphology of bacteria at the MIC after 120 min is consistent with the forward and side light scatter differences shown in Fig. 3.

**DISCUSSION**

We have previously shown that the loop region of human lactoferrin contains an amphipathic α-helical region exposed on the outer surface of lactoferrin on helix 1 and that a synthetic 11-amino-acid peptide from this region, HLP 2, has bactericidal activity towards E. coli (34). It has recently been reported that the homologous peptide from bovine lactoferrin adopted an alpha-helical structure in both trifluoroethanol and sodium dodecyl sulfate (25), indicating that hydrophobic conditions are required for maintenance of the alpha-helical structure. We have now demonstrated by using surface plasmon resonance that HLP 2 binds specifically to LPS of E. coli O111, showing the importance of this region to binding and antimicrobial pathogenesis. Previous evidence, obtained by using recombinant human lactoferrin mutated at residues 28 to 34, has shown that this region on the whole molecule is involved in binding to LPS (12). However, other sites may also play a role in binding, such as the arginine cradle proposed by Mann et al. (28), in which arginines 2, 3, and 4 at the amino terminus, in combination with the cationic region on helix 1 and that a synthetic 11-amino-acid peptide from this region, HLP 2, has bactericidal activity towards E. coli (34). It has recently been reported that the homologous peptide from bovine lactoferrin adopted an alpha-helical structure in both trifluoroethanol and sodium dodecyl sulfate (25), indicating that hydrophobic conditions are required for maintenance of the alpha-helical structure. We have now demonstrated by using surface plasmon resonance that HLP 2 binds specifically to LPS of E. coli O111, showing the importance of this region to binding and antimicrobial pathogenesis. Previous evidence, obtained by using recombinant human lactoferrin mutated at residues 28 to 34, has shown that this region on the whole molecule is involved in binding to LPS (12). However, other sites may also play a role in binding, such as the arginine cradle proposed by Mann et al. (28), in which arginines 2, 3, and 4 at the amino terminus, in combination with the cationic region on helix 1, form a positively charged cluster on the outer surface of human lactoferrin. The HLP 2 region is likely to play two roles in vivo, first within the whole human lactoferrin molecule and second as the peptide fragment lactoferricin, liberated after enzymatic degradation of lactoferrin. However, the mechanisms of action of lactoferrin and lactoferricin may well be different, as the free

**TABLE 2. E. coli fluorescence in the presence of HLP 2 and CCCP or in the presence of HLP 2 at 4°C determined by using DiBAC₄(3) and PI**

<table>
<thead>
<tr>
<th>Condition</th>
<th>Without HLP 2</th>
<th>With HLP 2</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>DiBAC₄(3)</td>
<td>PI</td>
</tr>
<tr>
<td>CCCP</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Absent</td>
<td>6.7</td>
<td>3.3</td>
</tr>
<tr>
<td>Present</td>
<td>46.0</td>
<td>54.2</td>
</tr>
<tr>
<td>Temp (°C)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>37</td>
<td>6.4</td>
<td>1.2</td>
</tr>
<tr>
<td>4</td>
<td>24.7</td>
<td>15.5</td>
</tr>
</tbody>
</table>

a E. coli grown to log phase was used to give a starting inoculum of 10⁶ bacteria/ml and incubated for 2 h in the presence or absence of HLP 2 and either with or without CCCP or at 4 or 37°C.

b Fluorescence was monitored by flow cytometry and is expressed as the percentage of fluorescent cells in the whole population. Results are means from at least four experiments.
peptide fragment has enhanced antibacterial activity compared to lactoferrin (4). Although the bactericidal actions of lactoferrin and lactoferricin may differ, the HLP 2 LPS recognition site probably plays an important role in initial bacterial-protein–peptide interactions.

The orientations of the charged amino acids play an important part in the ability of HLP 2 to exert its antimicrobial activity, as seen by the large reduction in antimicrobial activity when proline is substituted for methionine. This substitution was predicted, by molecular modelling, to disrupt the helix and therefore the orientations of charged amino acids (8). Conformational changes occur within each lobe of human lactoferrin upon binding of iron, when the two domains of each lobe come together, with major structural changes occurring around the hinge region and subtle differences occurring throughout the rest of the molecule (18). The differences in charge orientation at the HLP 2 region on helix 1 between the apolactoferrin and hololactoferrin could account for the inability of the holo form

FIG. 2. (a) Percentages of particles present after incubation for 2 h of E. coli O111 (NCTC 8007) with various concentrations of HLP 2 compared to that for the control (containing no HLP 2), measured by using flow cytometry. (b) Percentages of particles after incubation of E. coli NCTC 8007 with HLP 2 at the MIC for various time intervals compared to that for control samples containing no HLP 2. Experiments were repeated at least three times, with a starting inoculum of 10^8 bacteria/ml. Error bars indicate standard errors.

FIG. 3. Typical side and forward light scatter of control bacteria (black) and bacteria incubated with HLP 2 for 2 h (grey).
to exert antimicrobial activity (3). It has already been established that the affinity of apo human serum transferrin for the human transferrin receptor is lower than that of the diferric form, and in addition, subtle changes in the conformation of the diferric protein, as a result of a mutation close to one of the metal binding sites, reduce its affinity for the receptor (16).

We have used flow cytometry to investigate how HLP 2 exerts its action on E. coli O111 with the aid of specific markers. Exposure of the bacteria to HLP 2 results in the collapse of membrane potential, leading to pore formation, as shown by PI fluorescence, and causing a collapse in membrane integrity. This is followed by distortion of the cell morphology, as shown by the forward and side light scatter, which finally results in cell lysis. Lysis results in the release of proteases, which could lead to partial proteolysis of the peptide and a reduction in its effective concentration. As the D form of the peptide is likely to

FIG. 4. Electron micrographs of E. coli O111 NCTC 8007. (a) Control (magnification, ×3,600); (b and c) after 2 h of incubation with HLP 2 at the MIC (magnifications, ×3,600 [b] and ×7,200 [c]); (d and e) after 2 h of incubation with HLP 2 at half the MIC (magnification, ×13,500). The original inoculum size was 10^8 bacteria/ml. Arrows indicate separation of the outer membrane.
be more resistant to proteolysis, this could explain the enhanced potency of HLP 7, the D form of HLP 2, against *E. coli* O111, a *Klebsiella* strain, and *P. stuartii*. From the results of experiments carried out either in the presence of the metabolic inhibitor CCCP or at 4°C, we can conclude that the peptide acts at the membrane surface and not at the metabolic level. The results of our studies using flow cytometry indicate that HLP 2 exerts its antimicrobial effect by a mechanism similar to that of other cationic peptides which act at the bacterial cell membrane. The proposed mechanism by which cationic peptides exert their antimicrobial activity is by interacting with negatively charged divalent cation binding sites on the surface LPS, disrupting these sites and leading to uptake of peptide across the outer membrane. The affected membrane is thought to form channels which allow leakage of cytoplasmic molecules and lead to cell death (20). The differences between HLP 2 and other natural cationic peptides (36) are the comparatively low potency and relatively slow mode of action of HLP 2. We are therefore able to study how the peptide may act and thus gain insight into how HLP 2 and other alpha-helical cationic peptides may exert their activities. The timing of collapse of membrane potential (10 min), collapse of integrity (30 min), and cell lysis (within 2 h) suggests that HLP 2 initially attaches to a site on the LPS, leading to an interaction between the peptide and outer membrane (OM). This is further supported by the TEM micrographs, in which the OM was observed to detach at specific cell sites, either at the point of division or at the peripheral ends of the cells, without loss of structural rigidity. Differences in LPS concentration at different points along the OM could account for the specific detachment. The TEM results obtained in this study are different from those previously shown for lactoferrin (13) or lactoferricin (38), which cause electron-dense blisters, indicating that lactoferrin or lactoferricin simply binds at the cell surface, without penetration, whereas HLP 2 enters the OM.

The increased potency against *S. aureus* compared with that against *E. coli* can be explained by differences in the structure of the bacterial cell wall. It is likely that HLP 2 has a different mechanism of action towards *S. aureus* than towards *E. coli* because of the absence of LPS. The difference between the activities of HLP 1 and HLP 2 against *S. aureus* could be explained by differences in peptide size, allowing uptake of HLP 2 rather than HLP 1, but it is more likely to be due to differences in flexibility and conformation if the peptide binds to the peptidoglycan. This would also explain why HLP 6 still has activity against *S. aureus*. Differences in the composition of the bacterial cell wall and, in the case of gram-negative bacteria, differences in the structure of LPS which prevent bacterial interactions could account for the observations that certain bacteria are resistant to HLP 2 and for the differences in activity between those bacteria which are susceptible to HLP 2. In vivo, this may be important in establishing a bacterial flora.

We have been able to propose for the first time a mechanism of action for how the loop region of helix 1 in human lactoferrin, both in the whole protein and as a liberated peptide, can exert its antimicrobial activity. We have shown that both the helix and the charge are important for antimicrobial activity against *E. coli* and that subtle differences in charge orientation can cause significant differences in potency. The differences in potency between the free peptide and lactoferrin can be explained by the degree to which the helix can interact with the bacterial membrane as a result of the increased flexibility of the free peptide, to cause bacterial cell wall disruption.

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