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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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.

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 atoms of iron (35), an essential bacterial nutrient, and is restricted to the apoprotein, with the diferric 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 a surface helix on lactoferrin distinct from the area of iron binding (34) and in the region of LPS binding (12). We have shown that a synthetic 11-amino-acid peptide (previously designated HLT 2 [34] and now renamed human lactoferrin peptide [HLP] 2), which includes this LPS binding site, has potent antibacterial activity against *Escherichia coli* (34). The RKVR region found within HLP 2 forms the glycosaminoglycan binding site of human lactoferrin (28).

A number of other naturally occurring peptides have been shown to have antibacterial activity. These include magainins (39) from frogs, cecropins (21) from insects, and mellitin (19) from bee venom. Although these peptides have little sequence homology, they have two distinguishing features: a high content of basic amino acids, such as arginine and lysine, and the ability to adopt secondary conformations (namely, α-helices and β-sheet structures) in which there is a hydrophilic face of polar and positively charged residues on an axial plane (20). However, the three-dimensional structures of these peptides are not always apparent, as is the case with mellitin, which takes up a different structure in aqueous solution when self-association reactions take place (monomer to tetramer) (37), or with magainin when bound to the phosphatidylserine vesicles (33). Cationic peptides have been shown to form pores in artificial membranes (9, 11, 23), and they can also have specific binding sites on the outer membranes of bacteria (10). Their antimicrobial activity is likely to be determined by the mode of interaction with the bacterial cell membrane (20).

A structural feature of the human lactoferricin loop is the 11-amino-acid α-helical region with an asymmetric cluster of basic amino acids (34). In this study the structure-function relationships of the lactoferricin-related peptides are considered. Synthetic peptides homologous to this region and analogs were synthesized, and their antimicrobial activities against a

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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-FQWQRNMRKVRGPPVS-COOH), HLP 2 corresponded to the alpha-helical region of the loop in its native conformation (residues 20 to 30; NH2-FQWQRN MRKVR-COOH), HLP 6 had a proline substituted for methionine in HLP 2 (NH2-FQWQRNPRKVR-COOH), 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. Clinical 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. Peptides 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 10^8 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 was diluted 1:100 with fresh medium and incubated at 37°C to log-phase growth. Bacteria were washed in fresh medium, and cells were harvested to give a density of 10^8 cells/ml. Bacteria were incubated in different concentrations of HLP 2 in proteose peptone, and aliquots were withdrawn after 2 h of incubation at 37°C to give a dose-response assay. Time-response assays were performed at the MIC, with aliquots taken for analysis immediately (time zero) and at 15, 30, 60, 90, and 120 min. Bacteria were centrifuged at $13,400 \times g$ for 30 s and resuspended in fresh medium before flow cytometry. Bacterial membrane potential was determined by using the lipophilic anionic membrane potential-sensitive dye bis-1,3-(dibutylbarbituric acid)trimethine oxonol [DiBAC4(3)] (Molecular Probes, Inc.) (30) at a final concentration of 10 μg/ml. Membrane integrity was assessed with the cationic nucleic acid binding fluorescent dye propidium iodide (PI) (Sigma, Poole, United Kingdom) (17) at a final concentration of 10 μg/ml. Fluorescence was measured with an HS Bryte flow cytometer (Bio-Rad, Hemel Hempstead, United Kingdom) and compared to that of control bacteria. Results are the means from four separate experiments.

Metabolic activity. To investigate the effects of low temperature, cultures of *E. coli* NCTC 8007 were grown to log phase in 1% proteose peptone at 37°C, the temperature was reduced and equilibrated to 4°C, and the cultures were treated with HLP 2 at the MIC for 2 h. Samples were spun at 13,000 rpm for 30 s at 4°C, and the resulting pellet was resuspended in fresh cold medium. Parallel samples with *E. coli* NCTC 8007 incubated at 37°C in the presence of HLP 2 and control samples at 4 and 37°C without HLP 2 were also prepared. Aliquots of each sample were taken and subjected to flow cytometry in the presence of either DiBAC4(3) or PI. To determine the effect of the respiratory poison carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) (Sigma) (26) on the action of HLP 2, bacterial samples were treated with 50 μM CCCP with or without the peptide for 10 min at 37°C, and the cells were washed by centrifugation at $13,400 \times g$ for 30 s and resuspended in fresh medium. Aliquots of each sample were taken, and either DiBAC4(3) or PI was added for flow cytometric analysis.

Effect of HLP 2 on bacterial lysis and cell morphology. Bacterial lysis was assessed by reduction in particle number. Cell morphology was assessed by comparing forward and side light scattering of bacteria incubated with peptide at the MIC for 2 h with that of control cultures. TEM was employed to confirm morphological changes of the bacterial cells. Log-phase *E. coli* (approximately 10^8 bacteria/ml) incubated for various times in 1% proteose peptone with HLP 2 and control cultures were centrifuged at $4,000 \times g$ for 5 min, prefixed in 2.5%

TABLE 1. MICs of human lactoferrin peptides against a range of bacteria[a]

Species and strain	MIC (μM)[b]			
	HLP 1	HLP 2	HLP 6	HLP 7
E. coli NCTC 8007	500	290	>1,000	18
E. coli NCTC 10418	500	579	750	40
S. aureus NCTC 10571	500	14	250	14
Clinical isolates				
Acinetobacter sp.	2	0.86	10	0.3
E. aerogenes	>1,000	290	>1,000	>1,000
Klebsiella sp. strain 3105	>1,000	290	>1,000	80
P. stuartii	>1,000	>1,000	>1,000	110
P. mirabilis	>1,000	>1,000	>1,000	>1,000

[a] Bacteria were grown to log phase and incubated against twofold serial dilutions of HLP 1, HLP 2, HLP 6, or HLP 7 at 37°C overnight in 96-well microtiter plates.
[b] Determined by A_{620}.

glutaraldehyde in 0.2 M phosphate buffer (pH 7.3), postfixed in 0.5% osmium tetroxide in Millonigs constant-osmolarity phosphate buffer (pH 7.4), and dehydrated in graded ethanol solutions. Pellets were embedded in medium Taab Resin and polymerized for 24 h at 60°C. Sections (50 nm) were stained with aqueous uranyl acetate and lead citrate and examined with a Hitatchi H700 transmission electron microscope.

HLP 2 binding to LPS. The specificity with which peptide HLP 2 bound to LPS was measured by using surface plasmon resonance on a Biacore X (Pharmacia Biosensor AB). This measures the change in refractive index, shown as resonance units, of receptor binding to ligands on a chip surface (27). *E. coli* serotype O111 LPS (Sigma) was dissolved in phosphate-buffered saline (PBS) (pH 7.4) and equilibrated to saturation over the surface of a hydrophobic HPA sensor chip (Pharmacia Biosensor AB), to which it binds by hydrophobic interactions. Following saturation, peptide at various concentrations was suspended in PBS (pH 7.4) and passed over the coated chip, and peptide binding was measured as a change in resonance units by using BIA software. This was subtracted from binding of the peptide on a blank hydrophobic surface to give the number of resonance units after nonspecific binding had been taken into account. Experiments were carried out three times.

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 the *Acinetobacter* strain and showed activity against *E. aerogenes* and a *Klebsiella* strain but had no activity against *P. mirabilis* and *P. stuartii*. HLP 6, 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 dose-response assay (Fig. 1a) demonstrated that the addition of HLP 2 to *E. coli* NCTC 8007 results in 50% fluorescence at approximately 100 μM (0.15 mg/ml), and in the presence of PI at approximately 140 μM (0.2 mg/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
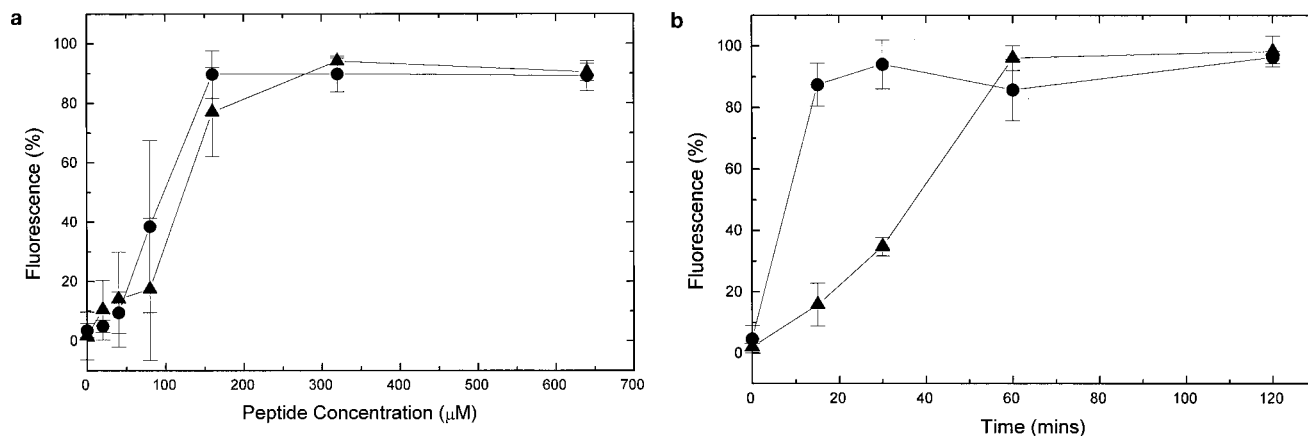
FIG. 1. Dose-response (a) and time-response (b) assays, by flow cytometry, of a starting inoculum of 10^8 E. coli O111 (NCTC 8007) cells per ml versus HLP 2. The percentages of fluorescent bacterial cells in the presence of PI (▲) or DiBAC$_4$(3) (●) are shown. Results are means ± standard errors from at least three experiments.

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 DiBAC$_4$(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 nonspecific 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 lactoferricin 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, 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 DiBAC$_4$(3) and PI

| Condition[a] | Fluorescence[b] | | | |
| | Without HLP 2 | | With HLP 2 | |
	DiBAC$_4$(3)	PI	DIBAC$_4$(3)	PI
CCCP				
Absent	6.7	3.3	98.3	99.4
Present	46.0	54.2	90.2	95.9
Temp (°C)				
37	6.4	1.2	96.3	98.2
4	24.7	15.5	94.1	97.2

[a] E. coli grown to log phase was used to give a starting inoculum of 10^8 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.
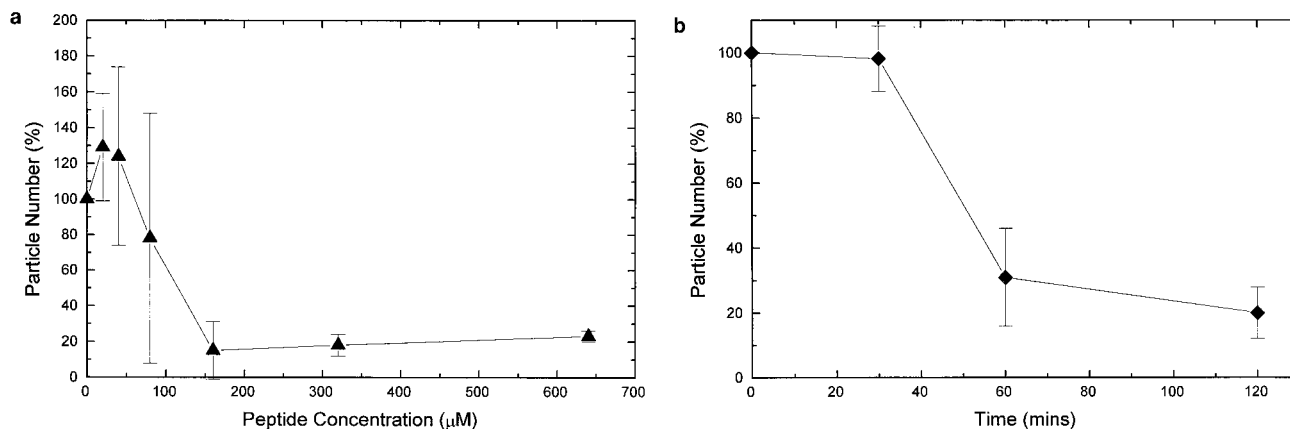
FIG. 2. (a) Percentages of particles present after incubation for 2 h of *E. coli* O111 (NCTC 8007) with various concentrations of HLP 2 compared to that for the control (containing no HLP 2), measured by using flow cytometry. (b) Percentages of particles after incubation of *E. coli* NCTC 8007 with 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.

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
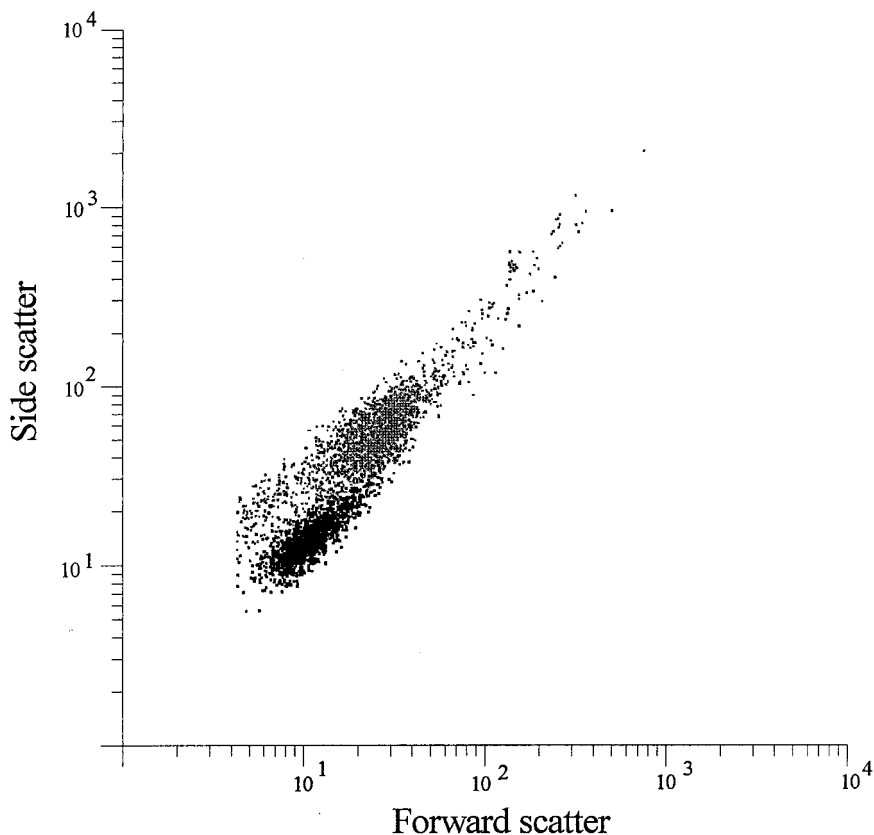

FIG. 3. Typical side and forward light scatter of control bacteria (black) and bacteria incubated with HLP 2 for 2 h (grey).
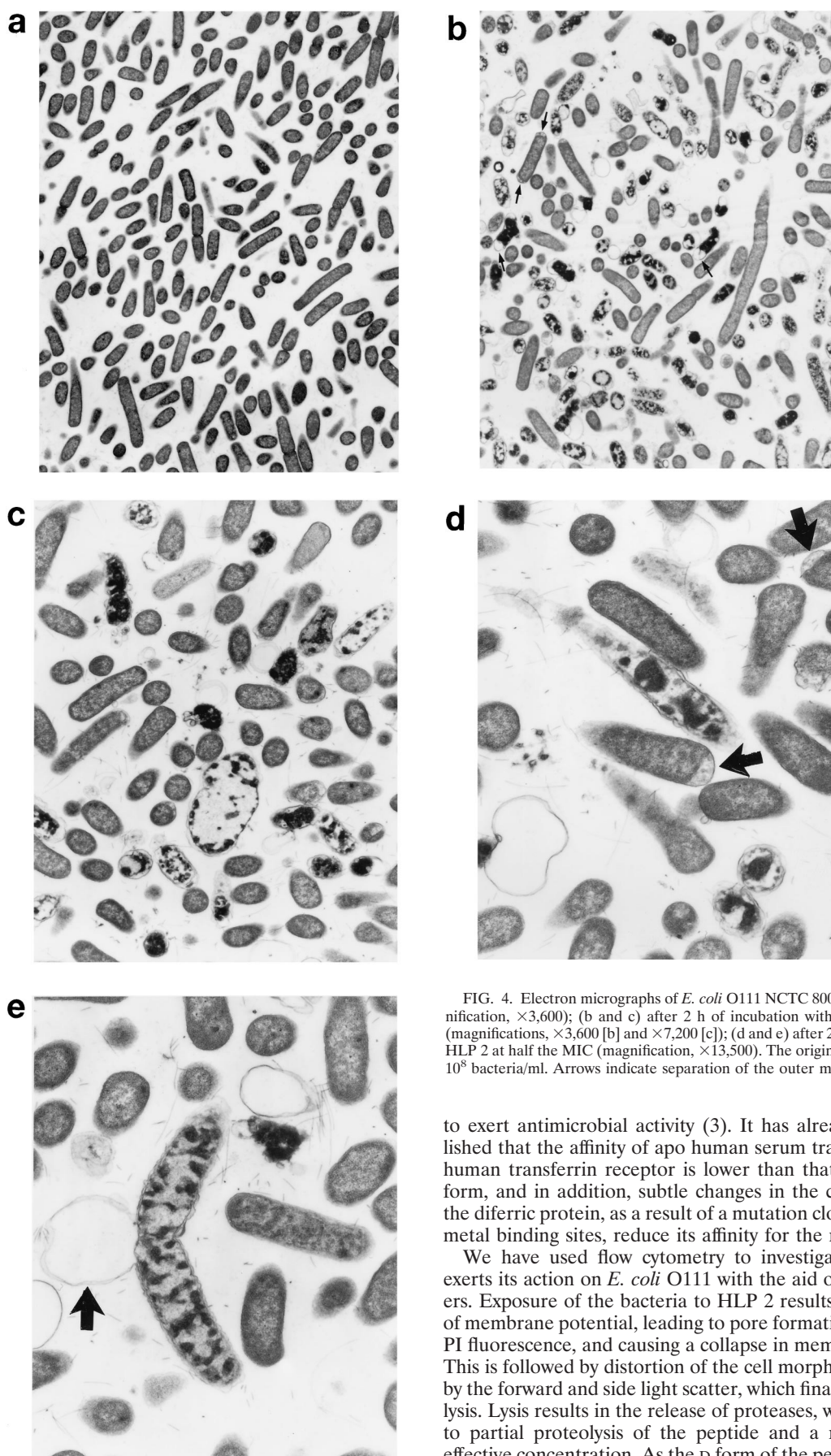
FIG. 4. Electron micrographs of *E. coli* O111 NCTC 8007. (a) Control (magnification, ×3,600); (b and c) after 2 h of incubation with HLP 2 at the MIC (magnifications, ×3,600 [b] and ×7,200 [c]); (d and e) after 2 h of incubation with HLP 2 at half the MIC (magnification, ×13,500). The original inoculum size was 10^8 bacteria/ml. Arrows indicate separation of the outer membrane.

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

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 these 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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