A Helix-Loop-Helix Peptide at the Upper Lip of the Active Site Cleft of Lysozyme Confers Potent Antimicrobial Activity with Membrane Permeabilization Action*

Received for publication, July 6, 2001, and in revised form, September 13, 2001

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*This work was supported in part by Research Grant C-2 13660129 from the Ministry of Education, Science, Sports and Culture of Japan. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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** Supported by the Japan Society for the Promotion of Science.

Recently, we have found that partially unfolded lysozyme exerts broad spectrum antimicrobial action in vitro against Gram-negative and Gram-positive bacteria independent of its catalytic activity. In parallel, an internal peptide (residues 98–112) of hen egg white lysozyme, obtained after digestion with clostripain, possessed broad spectrum antimicrobial activity in vitro. This internal peptide is part of a helix-loop-helix domain (87–114 sequence of hen lysozyme) located at the upper lip of the active site cleft of lysozyme. The helix-loop-helix (HLH) structures are known motifs commonly found in membrane-active and DNA-binding proteins. To evaluate the contribution of the HLH peptide to the antimicrobial properties of lysozyme, the HLH sequence and its secondary structure derivatives of chicken and human lysozyme were synthesized and tested for antimicrobial activity against several bacterial strains. We found that the full HLH peptide of both chicken and human lysozymes was potently microbialid against both Gram-positive and Gram-negative bacteria and the fungus Candida albicans. The N-terminal helix of HLH was specifically bactericidal to Gram-positive bacteria, whereas the C-terminal helix was bactericidal to all tested strains. Outer and inner membrane permeabilization studies, as well as measurements of transmembrane electrochemical potentials, provided evidence that HLH peptide and its C-terminal helix domain kill Gram-negative bacteria by crossing the outer membrane via self-promoted uptake and causing damage to the inner membrane through channel formation. The results are discussed in terms of proposed mechanisms for the catalytically independent antimicrobial activity of lysozyme that offer a new strategy for the design of potential antimicrobial drugs in the treatment of infectious diseases.

Lysozyme (1,4-β-N-acetylmuramidase) belongs to the class of enzymes that lyse the cell walls of bacteria as the bond between the C-1 of N-acetylmuramic acid and the C-4 of N-acetylglycamin of the peptidoglycan is cleaved (1). Lysozyme is an antimicrobial enzyme widely distributed in nature and is found in mammalian tissues and secretions, insects, plants, bacteria, and viruses (1). Besides its antimicrobial activity, lysozyme has many other functions including inactivation of certain viruses (2), surveillance of membranes of mammalian cells (3), enhancing phagocytic activity of polymorphonuclear leukocytes (4) and macrophages (5), stimulation of monocytes (6), antitumor activity (7), and induction of fusion of phospholipids vesicles (8).

The antimicrobial activity of lysozyme is known to be directed against certain Gram-positive bacteria (2, 9, 10) and to lesser degree in vitro against Gram-negative bacteria (2). Lysozyme, however, has the capacity to neutralize and strongly interact with lipopolysaccharides of Gram-negative bacteria (11). Moreover, its secretion in many tissues is known to be induced upon bacterial infection (12, 13). The importance of lysozyme as a key molecule in the defense against bacterial infection has been recognized (10, 14–16); however, it is still not certain whether or not its catalytic activity contributes to its antimicrobial function. The literature abounds with evidence implicating an alternative functional site in lysozyme having the primary bactericidal activity, with the N-acetylmuramidase activity of secondary importance in microbial defense (17, 18).

In previous studies (19, 20), we showed that the antimicrobial action of hen egg white lysozyme (cLz)3 depends not on its enzymatic activity but rather on a structural phase transition. Partially unfolded cLz showed enhanced bactericidal action against Gram-negative bacteria while retaining its inherent microbialid activity to Gram-positive ones (21, 22). Our results indicated that the bactericidal cLz undergoes conformational changes particularly at the aspartylglycyl sequences (residues 101 and 102) located in the loop structure at the upper lip of the active site cleft (19, 22, 23). This observation, together with the results of the proteolytic digestion of cLz, suggests that a specific bactericidal domain may actually be involved in the antimicrobial action of lysozyme. The digestion of cLz by clostripain produces a pentadecapeptide (residues 98–112) with bactericidal activity against Gram-positive and -negative bacteria (24). Of particular interest is that the pentadecapeptide forms

1 The abbreviations used are: cLz, chicken egg-white lysozyme; hLz, human lysozyme; HLH, helix-loop-helix/helical hairpin; CP-, peptide derived from chicken egg-white lysozyme sequence; HP-, peptide derived from human lysozyme sequence; cu, colony-forming units; ONPG, o-nitrophenyl-β-D-galactoside; diS-C3-(5), 3,3′-diisothiocyandis carbocyanine iodide; OM, outer membrane; IM, inner membrane; PADAC, 7-[(thiethyl-2-acetamido)-3-(2,4-di(N-methylamino phenylazo)-pyridinium-methyl)-3-cyhex-4-carboxylic acid; TSB, trypticase soy broth.
part of a helix-loop-helix (HLH) motif (residues 88–114 of cLz and 87–115 of human lysozyme, hLz) located at the upper lip of the active site cleft of the molecule. The helical hairpin structures, such as HLH observed in cLz (residues 88–114) and hLz (residues 87–115), are known motifs commonly found in bactericidal and cytolytic pore-forming proteins (25, 26). For example, cercopin A and B1, major antimicrobial peptides in the immune response of silk moths, consist of two α-helices flanked by a loop maintained by Gly and Pro residues (26–28). Colicin E1, a bactericidal plasmid-encoded protein with the ability to form an ion channel in the Escherichia coli cytoplasmic membrane, possesses a C-terminal α-helical hairpin that mediates insertion and subsequent channel assembly into the membrane (29–31).

Structure examination of lysozyme indicates that there are recurrent features around its catalytic apparatus. It is likely that interaction with substrate (peptidoglycan) or the bacterial surface (lipopolysaccharides) and the positioning of its HLH domain near the membrane surface appear to be key elements in the mode of bactericidal action of this enzyme. To explore the structure-antimicrobial relationship and to test this mechanism of action of lysozyme, we sought to elucidate the role of HLH domain on the antimicrobial action of lysozyme. In this investigation, the full HLH domain and several secondary structure derivatives of both hen egg white and human lysozyme were synthesized and tested for antimicrobial activity against different microorganisms. The synthetic HLH peptides and their fragments were also evaluated with regard to secondary structure elements and their ability to interact with and permeabilize the membranes of E. coli and model phospholipid membranes. The importance of this highly conserved sequence of HLH domain for antimicrobial specificity, the mechanism of action of known lysozyme molecules, and its potential as a new drug against bacterial infectious diseases are discussed.

**EXPERIMENTAL PROCEDURES**

**Materials**—The following polypeptides were synthesized by different companies: polypeptide DITASVNCARKIVSDGNGMNAWAVWRNR was from Respektor (St. Louis, Missouri); DIIADAVAKRVRDPQGIRAWAVWRNR, DIIADAVACRVRVDRPQGIRDPQGIR, and RAWAVWRNR were from Alpha Diagnostic (San Antonio, TX); DNIADAVACRVRVDPQGIRAWAVWRNR, DIIADAVACRVRVDRPQGIRDPQGIR, and RAWAVWRNR were from the peptide synthesis service of the University of Lausanne, Switzerland. Nigericin was from Wako Pure Chemicals (Osaka, Japan). 7-(Thiethyl-2-acetamido)-3-(2,4N,N-dimethylaminophenylazo)-pyridinium-methyl-3-cephem-4-carboxylic acid (PADAC) was from Calbiochem. Phospholipids were from the Institute of Bacteriology of the Veterinary Hospital, Zürichland.

**Antibacterial Assay—**Bactericidal assay was performed as described previously (24). Briefly, 50 ml of trypticase soy broth (TSB) was inoculated with a single colony of bacteria and incubated overnight at 37 °C. One ml of bacterial suspension was diluted (1:50) in TBS. Bacteria were grown at 37 °C until logarithmic phase was reached (~1–4 × 10⁶ cfu/ml). Bacteria were harvested by centrifugation at 2000 × g for 10 min, washed, and resuspended (10⁶ cfu/ml) in a 10 mm sodium phosphate buffer, pH 7.4. Aliquots (50 μl) of the bacterial suspension were mixed with 50 μl of water containing the test peptide in 2-fold serial dilutions. 100 μl of 2% TSB in 10 ml sodium phosphate buffer was then added. The mixture was incubated at 37 °C for 2 h, serially diluted in sodium phosphate buffer, pH 7.4, and plated on tryptose soy agar. Colony-forming units were obtained after incubation of the plates at 37 °C for 24 h. Assays were performed in duplicate.

**Candidacidal Assay—**Susceptibility of C. albicans was tested essentially according to the procedure described above for the antibacterial assay. Briefly, 50 μl of 10⁶ cfu/ml of C. albicans blastoconidia derived from a 24-h culture in TSB were incubated with 50 μl of the test peptide solution and 100 μl of 2% TSB in phosphate buffer. After an incubation time of 2 h, the mixture was plated on TSB agar. The plates were incubated for 24 h at 37 °C, and the colonies were counted.

**Outer Membrane Permeability—**Outer membrane permeabilization of E. coli ML-35p was determined by measuring the release of β-lactamase activity into the culture medium using PADAC as a substrate, according to the procedure described by Lehrer (33). Bacteria grown to logarithmic phase were collected, washed, and resuspended (10⁶ cfu/ml) in 10 mm sodium phosphate buffer, pH 7.4. Bacterial membranes (50 μl) were incubated with predetermined preheated cuvette to 37 °C containing 612.5 μl of 10 mM sodium phosphate buffer, pH 7.4, 1% TSB, and 12.5 μl of 2.7 mM PADAC. After incubating at 37 °C for 15 min, test peptide solution (50 μl) was added (0.5 μM). The permeabilization of outer membrane was monitored by measuring the decrease of absorbance at 570 nm over time due to PADAC hydrolysis. Controls contained water instead of peptide solutions.

**Inner Membrane Permeability—**Determination of the inner membrane permeabilization was performed by measuring the β-galactosidase activity of lactose permease-deficient E. coli ML-35p at 37 °C using ONPG as a substrate (33). An aliquot (75 μl) of bacteria suspension (10⁶ cfu/ml) was pipetted into a 37 °C preheated cuvette, containing 550 μl of 10 mM sodium phosphate buffer, pH 7.4, 1% TSB, and 75 μl of 25 mM ONPG. After 15 min of incubation, 50 μl of the test peptide solution was added (0.5 μM). The production of β-nitrophenol over time was monitored spectrophotometrically at 400 nm. An equivalent volume of water replaced the peptide solution in the control assay.

**Preparation of Liposomes—**Small unilamellar vesicles were prepared from phospholipids of E. coli K-12, as described previously (34, 35). An aliquot (100 μl) of pure phospholipid stock (50 mg/ml argon-saturated 2 ml 2-mercaptoethanol) was added to 900 μl of 10 mM sodium phosphate buffer, pH 7.4, and 1% TSB. The production of β-nitrophenol over time was monitored spectrophotometrically at 400 nm. An equivalent volume of water replaced the peptide solution in the control assay.

**Membrane Potential (ΔΨ) of E. coli Phospholipid Liposomes—**The ability of peptide to dissipate membrane potential (ΔΨ) was determined by measuring the fluorescence quenching of [diS-C₃(5)] using a Hitachi F-3000 fluorescence spectrophotometer (Hitachi, Tokyo, Japan), as reported previously (34, 35). Experiments were performed at 25 °C, excitation at 622 nm, and recording emission at 670 nm. The reaction mixture contained 50 mM sodium phosphate, pH 7.5, 1 mM diS-C₃(5), and 70 μM liposomes (K⁺-loaded) in a total volume of 1.0 ml. The K⁺-loaded liposomes were energized (inside negative, −160 mV) by a valinomycin-mediated potassium diffusion potential. Peptide was then added at the indicated concentrations, and changes in fluorescence were monitored over time. The data were compared with that of the control experiment of the nigericin-mediated electrodineutral exchange of K⁺ for H⁺. To verify the integrity of treated liposomes during assay, nigericin was added after 650 s. The results are presented as fluorescence intensity.

**Liposome Binding—**Peptides (final concentration of 2 μM) were dissolved in 1 ml of phosphate-buffered saline buffer, pH 7.3. A 10-μl aliquot of the liposomes preparation in the same buffer was added to give a final concentration of 500 μg of phospholipid/ml (147 μM) and then excited at 288 nm with 2-nm resolution. Trytophan (Trp) intrinsic emission spectra were recorded from 300 to 420 nm and were corrected.
The view is ~45° from the right side to the face of the active site cleft. The α-helical hairpin (HLH) motif is indicated (dark region) at the upper side of the active site cleft. The two catalytic residues (Glu and Asp<sup>23</sup>) are shown. The ribbon structure was generated by RasMol software with the Protein Data Bank file of clz (code 1HEW). B, the amino acid sequence of the α-helical hairpin motifs of hen egg white and human lysozymes. The identical (large bold letters) and partially homologous (large letters) residues are shown. Note the fully identical amino acid sequence at the C-terminal helix of the HLH motif between clz and hLz.

for the blank values and dilution by addition of liposomes or additives such as EDTA.

Computer-assisted Structural Analysis—Three-dimensional structures of lysozyme and its HLH peptide were generated by RasMol version 2.6 (Biomolecular Structures Group, Glaxo Wellcome) using the Protein Data Bank file retrieved from Swiss-Prot data base of human (code 1LZS) and hen egg white (code 1HEW) lysozyme.

RESULTS

Structure and Location of the Helix-Loop-Helix of Lysozyme—Recently, we presented evidence that proteolytic digestion of lysozyme by clostripain releases an antimicrobial peptide (residues 98–112) from lysozyme that forms the middle part of an HLH motif (24). The three-dimensional fold of lysozyme when complexed with a substrate analogue (GlcNAc<sub>3</sub>) is shown in Fig. 1. The active site of e-type lysozyme divides the molecule into two domains, the α-domain and the β-domain, whereas the HLH domain (dark region, Fig. 1A) is uniquely located at the upper lip of the active site of the enzyme. The HLH domain falls within the region of greatest degree of conservation of all α-type lysozymes (36). The amino acid sequences of the HLH domain of clz and hLz are shown in Fig. 1B. Sixteen of 28 residues are identical, with 5 others being chemically conservative substitutions. Given this degree of homology and the unique spatial location at the active site cleft, this HLH domain might serve as the primary antimicrobial domain of lysozyme, particularly as its α-helical hairpin structure resembles those of the bactericidal motifs reported for colicins and cecropins (27, 31).

Design of the Synthetic Peptides—The three-dimensional structure of the HLH domain is shown in Fig. 2A. For this study, the full HLH domains of chicken (clZ-(87–114)) and human (hLz-(87–115)) lysozyme were systematically synthesized. To examine more closely the structural requirements needed for the antimicrobial action, the N-terminal helix (H1), C-terminal helix (H2), and the intervening loop (Lp) of the respective HLH domain were also synthesized (Fig. 2B). We observed that the C terminus of the loop in the peptide of hLz possesses a positively charged residue (Arg<sup>107</sup>), whereas in the clZ there is a neutral residue (Asn<sup>106</sup>). To ascertain the positively charged Arg<sup>107</sup> that might be essential for the bactericidal capacity of hLz, two analogues (HP-Lp and HP-Lp<sup>′</sup>) for the peptide loop of human HLH domain were synthesized with single Arg addition (HP-Lp<sup>′</sup>).

Bactericidal Activity—Chicken lysozyme and the synthetic peptides were tested for bactericidal activity against six Gram-negative (B. bronchiseptica, E. coli, K. pneumoniae, P. aeruginosa, S. marcescens, and S. typhimurium), six Gram-positive (B. subtilis, M. luteus, S. aureus, S. epidermidis, S. lentus, and S. zooepidemicus), and a fungus (C. albicans). As shown in Table I, lysozyme and the full HLH peptides of both clz (CP-full) and hLz (HP-full) were effective against almost all bacterial strains tested. However, HP-full peptide exerted a markedly stronger bactericidal activity against all test strains than that of CP-full peptide. The bactericidal activity of HP-full was also higher than that of lysozyme against almost all the bacterial strains investigated. Only against E. coli and S. marcescens was it less bactericidal than lysozyme. The peptide CP-full inhibited weakly the growth of E. coli and was devoid of antibacterial activity against S. aureus and C. albicans. A common antimicrobial behavior shared by the full HLH domain of clz (CP-full) and hLz (HP-full) is that they exert stronger effects against Gram-positive than Gram-negative bacteria. It is worthy to note that Gram-positive bacteria are much more susceptible to the action of HLH peptides than the Gram-negative ones. The peptides CP-full and HP-full can ideally be resolved into three secondary structural elements as follows: two α-helical structures (N- and C-terminal) and the loop connecting the two α-helices. To investigate which part of the HLH sequences of CP-full and of HP-full is essential for bactericidal activity, the peptides CP-(87–100) (CP-H1), CP-(101–106) (CP-Lp), CP-(107–114) (CP-H2), HP-(87–101) (HP-H1), HP-(102–106) (HP-Lp), HP-(102–107) (HP-Lp<sup>′</sup>), and HP-(107–115) (HP-H2) were synthesized and tested for bactericidal activity. The C-terminal α-helix HP-H2 and HP-H2 derived from the HLH sequence of clZ and hLz, respectively, exerted much stronger bactericidal activity than the N-terminal α-helix (CP-H1 and HP-H1) against all strains tested. The C-terminal α-helix (CP-H2), however, was inactive against S. marcescens. The N-terminal α-helix (CP-H1) was specifically bactericidal, although to different degrees, against all Gram-positive bacteria tested. In addition, it exhibited significant activity against C. albicans and was weakly effective against E. coli. In contrast to clZ, hLz-derived N-terminal α-helix (HP-H1) was bactericidal against only three Gram-positive bacteria (B. subtilis, M. luteus, and S. lentus) and the Gram-negative bacteria B. bronchiseptica. All of the loop sequences of the parent HLH domain derived from either clZ (CP-Lp) or hLz (HP-Lp and HP-Lp<sup>′</sup>) virtually exhibited strong bactericidal activity against B. subtilis. CP-Lp was essentially inactive against all the other strains tested but exhibited weak activity against C. albicans. HP-Lp showed a similar trend of activity but was moderately active against S. zooepidemicus and weakly active against S. typhimurium. The analogous loop derived from the human sequence with a C-terminal Arg residue (HP-Lp<sup>′</sup>) was weakly active against S. epidermidis and S. zooepidemicus and inactive against all of the other bacteria tested.
Candidacidal Activity—Chicken lysozyme and the synthetic peptides exhibited variable fungicidal activity against *C. albicans*. Lysozyme, the full HLH domain of hLz (HP-full) and its C-terminal /H9251-helix (HP-H2) showed a strong fungicidal activity. On the other hand, the full HLH domain (CP-full) of cLz was inactive against this fungus. However, the peptides CP-H1 (helix 1), CP-Lp (loop), and CP-H2 (helix 2) of the parent CP-full, exhibited a wide range of activity against *C. albicans*, ranging from weak to strong. CP-H2 was the most active and CP-Lp was the weakest.

Permeabilization of Outer and Inner Membranes—The most active bactericidal peptides against Gram-negative bacteria, including *E. coli*, were HP-full and its C-terminal helix HP-H2 derived from hLz as shown in Table I. The ability of lysozyme, F, A, ribbon representation of the fold of /H9251-helical hairpin (HLH) motif of cLz. The three-dimensional structure of /H9251-helical hairpin of hLz is exactly the same shown for cLz. The secondary structural elements are as follows: /H9251-helix 1 (H1), Asp87–Ser100; /H9251-helix 2 (H2), Ala107–Arg114; and loop (Lp), Asp101–Asn106. The tightness of the loop between the two helices is constrained by Gly102 and Gly104 (cLz sequence) and Pro103 and Gly105 (hLz sequence).

### Table I

Antibacterial activity of lysozyme, the synthetic helical-hairpin domains of chicken (CP-full) and human (HP-full) lysozyme, and their secondary structure derivatives

| Organisms | Strain | Activitya | Activityb |
|-----------|--------|-----------|-----------|
| **Gram-negative bacteria** | | | |
| *B. bronchiseptica* | WS | 1.6 | 9.0 | 0.0 | 0.3 | 2.2 | 0.5 | 0.0 | 0.0 | 0.9 |
| *E. coli* | ATCC 25922 | 2.0 | 0.1 | 0.1 | 0.0 | 0.3 | 2.0 | 0.0 | 0.0 | 0.7 |
| *K. pneumoniae* | ATCC 13883 | 2.0 | 0.1 | 0.1 | 0.0 | 0.3 | 2.0 | 0.0 | 0.0 | 0.7 |
| *P. aeruginosa* | ATCC 27853 | 1.0 | 0.4 | 0.0 | 0.0 | 0.3 | 2.0 | 0.0 | 0.0 | 0.7 |
| *S. marcescens* | ATCC 8100 | 1.6 | 0.4 | 0.0 | 0.0 | 0.3 | 2.0 | 0.0 | 0.0 | 0.7 |
| *S. typhimurium* | ATCC 14028 | 1.6 | 0.1 | 0.0 | 0.0 | 0.3 | 2.0 | 0.0 | 0.0 | 0.7 |
| **Gram-positive bacteria** | | | |
| *B. subtilis* | BGA | 2.6 | 2.8 | 0.9 | 1.2 | >3.0 | 0.7 | 2.0 | 2.3 | >3.0 |
| *M. luteus* | ATCC 4698 | 2.5 | >3.0 | 0.8 | 1.5 | >3.0 | 0.5 | 0.0 | 0.0 | 2.0 |
| *S. aureus* | ATCC 259230 | 0.2 | 0.5 | 0.0 | 0.8 | 3.3 | 0.0 | 0.0 | 0.0 | 2.3 |
| *S. epidermidis* | ATCC 12228 | 0.2 | 0.1 | 1.1 | >3.0 | >3.0 | 0.0 | 0.0 | 0.1 | >2.5 |
| *S. lento* | WS | 2.7 | 1.8 | 0.4 | 1.0 | >3.0 | 0.4 | 0.0 | 0.0 | >3.0 |
| *S. zoosporic* | ATCC 2091 | 0.7 | 0.6 | 0.4 | 0.8 | >3.0 | 0.6 | 0.4 | 1.1 | 1.1 |
| **Fungus** | | | |
| *C. albicans* | ATCC 2091 | 2.4 | 0 | 0.7 | 0.3 | 1.4 | 3.0 | 0 | 0 | 0.1 | 0 | 0.0 | 2.4 |

*a* Antibacterial activity is represented as log N/N, where N refers to the control number of colonies without antibacterial material, and N1 refers to the number of colonies containing antibacterial material after an incubation period of 2 h. The peptide concentrations were 25 × 10^{-9} mol per assay. Chicken lysozyme concentration was 83 × 10^{-9} mol per assay. WS, wild strain. The assays were performed in triplicate.

*b* From Pellegrini et al. (28).
HP-full, and HP-H2 to permeabilize the OM and IM of E. coli ML-35p was assessed by monitoring the release of intracellular β-lactamase (periplasmic) and β-galactosidase (cytoplasmic), respectively. As shown in Fig. 3A, lysozyme and the two peptides were able to permeabilize the OM and IM; however, the membrane permeabilization by lysozyme was more pronounced. The hydrolysis of PADAC (OM) and ONPG (IM) could be revealed 15 min after the addition of the peptides to E. coli ML35p. The kinetics of bactericidal activity by HP-full and HP-H2 was investigated against E. coli ML35p, the same strain used in OM and IM permeabilization. The bactericidal effect of lysozyme and both peptides was detectable after 15 min and increased in a time-dependent manner (Fig. 3B). After 90 min of incubation, the reduction of E. coli ML35p viability by HP-H2 was comparable with that yielded by lysozyme, but it was greater than the parent HP-full peptide. Reduction in colony-forming units of E. coli ML35p by HP-full (Fig. 3B) paralleled the damage to the IM (Fig. 3A).

**Dissipation of Lipid Membrane Electrochemical Potential by the Active Peptides**—The active bactericidal (CP-full, CP-H1, CP-H2, HP-full, and HP-H2) and inactive (CP-Lp, HP-H1) peptides were tested for their ability to interact with lipid bilayers and to dissipate the membrane potential. Dissipation of membrane potential was determined by using the fluorescence probe diS-C3-(5) and liposomes made from phospholipids of E. coli, as we described previously (35). Peptide (2 μM) was mixed with K⁺-loaded vesicles previously hyperpolarized (~160 mV) with valinomycin and the fluorescence diS-C3-(5) probe. Fluorescence recovery kinetics was monitored, and maximal recovery was determined by addition of nigericin after 11 min (Fig. 4). Lysozyme (cLz) showed a very weak effect on the electrochemical potential. Addition of the inactive peptides (CP-Lp and CP-H1) against E. coli (Table I) to hyperpolarized liposomes had little effect on the reversal of the fluorescence quenching. However, the active bactericidal peptides (CP-full, CP-H1, CP-H2, HP-full, and HP-H2) linearly reversed fluorescence quenching. Reversal of fluorescence quenching (dissipation of electrochemical potential) reflected the antimicrobial potency of the peptides. Dissipation of the membrane potential by the most strongly bactericidal peptides (HP-full, HP-H2, CP-full, and CP-H2) was considerably more effective than by the weakly bactericidal peptides (CP-H1 and HP-H1). Reversal of fluorescence quenching by peptides demonstrated their ability to cause leakage of the dye (positively charged) as a result of the depolarization of the vesicles. HP-H2, HP-full, and CP-H2 at a bactericidal concentration (2 μM) induced a collapse in membrane potential within 10 min as significant as 69, 65, and 61%, respectively, of the maximum fluorescence recovered by the addition of nigericin. A progressive collapse of membrane potential indicated ion channel formation.

**Binding Interface of the Active Peptides into Lipid Bilayer**—Tryptophan fluorescence is strongly influenced by the indole side chain and has proved to be a useful tool to monitor conformational changes in proteins and protein-membrane interactions (37). When buried in a hydrophobic environment, Trp fluorescence generally shifts to a shorter (blue) shift maximal wavelength (λmax) and often exhibits an increase in maximum fluorescence intensity (Fmax). An opposite effect is observed in a polar environment. To delineate.
the peptide-lipid binding interface of the membrane active peptides (CP-full, HP-full, and CP-H2), we relied on the two Trp residues, Trp$^{109}$ and Trp$^{111}$ (cLz sequence) and Trp$^{109}$ and Trp$^{112}$ (hLz sequence), as distinct reporters. This approach was facilitated by the natural occurrence of these two Trp residues in the helix 2 of the hairpin (Fig. 5E). The concentration of peptide was adjusted to 2 μM, and the Trp residues were excited at 288 nm. The emission spectra were recorded in the absence and presence of liposomes made of E. coli phospholipids. As seen in Fig. 5, A–C, in the presence of liposomes (+LP), cLz, CP-full, and HP-full showed quenching in $F_{\text{max}}$ and slightly shifted $\lambda_{\text{max}}$ (3 and 5 nm, respectively) to shorter wavelengths (blue shift) by addition of vesicles compared with the peptide alone. A similar effect of vesicles on the emission spectra of CP-H2 (Fig. 5D) and HP-H2 (data not shown) was observed. The results indicate that the peptides interact with liposomal membrane and that Trp residues are located at the membrane interface.

It is known that divalent cations play a specific structural role in stabilizing prokaryotic membranes as metal-ion bridges between phosphate groups of phospholipids (38). Most likely, the partitioning of Trp residues of the full HLH domain at the membrane surface is due to interaction with the Ca$^{2+}$ bridges of the membranes. To determine whether divalent cations play a role in membrane binding, the emission spectra of the peptides were recorded in the presence of EDTA (2 mM) and liposomes plus EDTA (Fig. 5). As shown in Fig. 5, B and C, addition of EDTA had no effect on the spectrum of the full HLH peptides alone (+EDTA). The spectra of the peptides with liposomes in the presence of EDTA (+EDTA, LP) increased the spectral intensity (Fig. 5). Interestingly, removal of Ca$^{2+}$ from membrane by EDTA (+EDTA, LP) induced a considerable increase in the emission (more buried Trp into lipid bilayer) of the helix 2 (Fig. 5D) but had a lesser effect on the parent peptide CP-full (Fig. 5B) or lysozyme (Fig. 5A).

The two Trp residues are located in the helix 2 (CP-full; Trp$^{109}$, Trp$^{111}$, and HP-full; Trp$^{109}$, Trp$^{112}$). The quenching of fluorescence (more exposed Trp) and blue-shifted $\lambda_{\text{max}}$ in the presence of liposomes (Fig. 5C) clearly indicated a direct association of the helical hairpin with lipid bilayer, although the indole side chains of Trp residues appear to be localized in the polar environment at the interface. Taken together, these data on membrane permeation in E. coli, polarized liposomes, and fluorescence quenching suggest that the amphipathic helix 1 of the helical hairpin (Fig. 5E) could account for membrane insertion that places the helix 2 (Trp-containing) close to the polar membrane interface.

**DISCUSSION**

Structural and functional characterization of natural antibacterial peptides derived from processed precursors is of growing interest because of their possible therapeutic applications. Studies conducted on various antimicrobial peptides tend to emphasize the role of α-helical structure and net positive charge for bacteriolytic activity. A major group of these antimicrobial peptides shares a common structural motif, namely a helix-loop-helix (HLH) or α-helical hairpin (25, 27, 31, 39), and are composed of short sequences (≤40 residues) that vary considerably in chain length, hydrophobicity, and overall charge distribution.

Lysosome is known to have a variety of folding topologies around the active site cleft and different saccharide-binding modes (41). A computer-assisted structural analysis of lysozyme, strikingly revealed an α-helical hairpin motif, commonly found in bactericidal and membrane active proteins (25, 26, 31, 39, 40), extending at the upper lip of the active site cleft of the enzyme (Fig. 1, dark region). As in the cecropins (26, 28, 39), the α-helical hairpin motif found in lysozyme consisted of two α-helices joined by a hinge region (loop) stabilized by a glycine-glycine doublet (cLz sequence, DGNG) or a proline-

**Fig. 5.** Tryptophan fluorescence reports liposome-induced changes in local environment of bactericidal peptides. Peptides tested were intact cLz (A), CP-full (B), HP-full (C), and CP-H2 (D). Peptide concentration was adjusted to 2 μM in 1 ml of phosphate-buffered saline, pH 7.3, and excited at 288 nm with 2-nm resolution, and Trp emission spectra were collected. Emission spectra were recorded in the absence (alone) and presence (+LP) of liposomes (147 μM total lipid) made from E. coli phospholipids in the same buffer. The emission spectra were also obtained in the presence of 2 mM EDTA (+EDTA). E, schematic ribbon representation of HLH domain of cLz showing the location of the two Trp residues (Trp$^{109}$ and Trp$^{111}$). Note the corresponding Trp residues of hLz are Trp$^{109}$ and Trp$^{112}$. Helix 1 (H1), helix 2 (H2), and loop (LP) regions are indicated.
cytoplasmic membrane. To reach this, the α-helix peptides must penetrate the OM in Gram-negative bacteria. The α-helical hairpin antimicrobial peptides derived from lysozyme were able to permeate the OM of *E. coli* ML-35p in a time-dependent manner, suggesting that they crossed the OM by a self-promoted uptake pathway (Fig. 3). HP-full appears to be more effective than HP-H2. Whereas the kinetic curves showing a bactericidal effect after 15 min was as expected, the bactericidal activity revealed after 90 min was not anticipated because HP-full was less efficient than HP-H2 with the *E. coli* strain ATCC 25922. This indicates that the two *E. coli* strains are affected differently by the bactericidal peptides.

The ability of full HLH domains and their C-terminal helices of cLz and hLz to dissipate the membrane potential of hyperpolarized liposomes indicates that the peptides rapidly disrupt the lipid bilayer most likely by forming an ion channel (Fig. 4). Strikingly, C-terminal helices (CP-H2 and HP-H2) dissipated the membrane potential more potently than their respective parent HLH peptides (Fig. 4). The ion channel seems to remain open and stable because the reversal of quenching was linear. This is consistent with the bactericidal action of defensins that form barrel-stave channels where the peptide molecules (staves) insert into the membrane and oligomerize to form a channel (barrel) (44–46). However, HP-full and HP-H2 were both able to permeabilize the IM to the larger (116 kDa) compound, cytoplasmic β-galactosidase (Fig. 3A), indicating large multimeric pore formation in the IM. Together, these observations indicate that peptides could induce disruption of the OM and IM, where the IM disintegration could result, as a common rule, in a lethal event. Permeabilization of IM can also be a sign of peptide passage to an internal target, and this would merit further investigation.

The two tryptophan residues of CP-full and HP-full (Fig. 5E) were shown to be located at the interface (quenching) upon association with the liposomal membrane (Fig. 5, B and C). Hence one can envision that the HLH peptide inserts into the lipid bilayer most likely via its N-terminal helix, whereas anionic head groups of phospholipids make direct contact with the C-terminal helix. Differences in overall peptide hydrophobicity between N-terminal (high hydrophobic) and C-terminal helices (low hydrophobic) support the conclusion that the N-terminal helix is the portion of the HLH domain to partition into the hydrophobic portion of lipid bilayer. In this context, CP-full or HP-full permeates the bacterial membrane by spanning the membrane via the N-terminal helix, likely forming a spiral-like channel, as an oligomer or stacks in the lipid bilayer. This model agrees well with the documented strong preference of Trp residues of membrane proteins to localize on the lipid-water interface, in particular at the trans-side of the membrane to control the topology of membrane proteins (47). A striking example of the enrichment of Trp at the interface is provided by the channel-forming peptide gramicidin A (48). This Trp-containing peptide is a monomer, cytoplasmic β-galactosidase (Fig. 3A), indicating a single transmembrane region, and oligomerizes upon insertion into the lipid bilayer to form a multimeric channel. Together, these observations indicate that peptides could induce disruption of the OM and IM, where the IM disintegration could result, as a common rule, in a lethal event. Permeabilization of IM can also be a sign of peptide passage to an internal target, and this would merit further investigation.

Two main mechanisms have been suggested for peptide permeation of the bacterial membrane as follows: (i) the barrel-stave mechanism, where bundles of peptides form transmembrane pores through the bacterial membrane, as is proposed for...
defensins (50); and (ii) the carpet-like mechanism, where membrane destruction and solubilization occur via parallel binding of the peptides to the bacterial membrane, covering the membrane in a carpet-like manner (51). The results of antimicrobial action, membrane permeabilization (Fig. 3), and liposome interaction (Fig. 5) of C-terminal helix (CP-H2 and HP-H2) support a carpet-like mechanism of action.

In conclusion, our study introduces a novel α-helical hairpin peptide within the c-type lysozyme sequence with a wide and very potent antimicrobial activity, which is among the highest reported for α-helical type antimicrobial peptides. The parent α-helical hairpin peptide of either Elz or Hz exhibited stronger antimicrobial capacity than its single helix peptides, the N-terminal and C-terminal helices. The single N-terminal helix was only active against Gram-positive bacteria, whereas the C-terminal helix was active against all microorganisms strains tested. The variable microbial susceptibility to the different domains of the α-helical hairpin peptide indicates that different bacteria selectively interact with different portions of the α-helical hairpin peptide. The results suggest a new strategy for the design of a repertoire of simple and easily manipulated α-helical antimicrobial peptides. Each α-helical peptide has a unique spectrum of activity (Table I). The existence of a peptide repertoire will allow the most efficient peptide to be chosen for a particular target cell. Acting separately or in concert, they may provide a better shielding against a wider range of infectious microbes. The susceptibility of a wide range of microbes to the α-helical hairpin and its C-terminal peptides was associated with membrane permeabilization and dissipation of membrane potential (Figs. 3 and 4). Therefore, bacteria might not so easily develop resistance to drugs that trigger such a destructive mechanism. Advantageously, the α-helical hairpin and its C-terminal peptides of chicken or human lysozyme did not show any lytic activity toward human red blood cells in up to 3-fold of concentrations used in antimicrobial assay (data not shown), hence heralding fascinating opportunities for therapeutic applications.

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