Toward Understanding the Cationicity of Defensins

ARG AND LYS VERSUS THEIR NONCODED ANALOGS

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Antimicrobial peptides (AMPs) constitute part of the first line of innate immune defense in vertebrates against infectious microbes (1). Most AMPs are cationic and kill microorganisms through permeabilization of the microbial membrane composed of negatively charged phospholipids such as phosphatidylglycerol and cardiolipin (2, 3). The selectivity of cationic AMPs toward microorganisms is further conferred by negatively charged molecules extruding out of the surface of the bacterial cell wall, i.e. teichoic acids in Gram-positive and lipopolysaccharides in Gram-negative strains of bacteria. It is believed that electrostatic interactions dictate not only the uptake of cationic AMPs across the bacterial cell wall but also their ability to permeabilize the cytoplasmic membrane and to induce leakage of cellular contents (4).

A large subfamily of cationic AMPs in humans is termed defensins (5–8). Human defensins are classified into α- and β-families on the basis of sequence homology and the connectivity of six conserved Cys residues. Lehrer and co-workers (9, 10) discovered in 1985 from the azurophilic granules of neutrophils the prototype α-defensins, also known as human neutrophil peptides or HNPs. To date, six human α-defensins, four primarily from neutrophils (HNP1–4) and two from intestinal Paneth cells (HD5–6) (11–14), and a number of β-defensins expressed predominantly in epithelial cells of various tissues and organs have been described (15–17). Despite the differences in size, cellular origin, disulfide topology, and amino acid composition, defensins from both families share a similar core structure of a three-stranded β-sheet stabilized by three intramolecular disulfide bridges (18–21). The pleiotropic roles of human defensins, manifested by their multifaceted antimicrobial and immunomodulatory functions in innate and adaptive immunity (22, 23), and their mechanisms of action at the molecular level have recently been the focus of intense scrutiny.

Sequence analysis of most known mammalian α-defensins indicates that Arg is strongly selected over Lys by a ratio of ~9:1, with few exceptions such as corticostatin/defensin-like peptides (RK-1 and RK-2) expressed in the rabbit kidney (24, 25). In humans, none of the six α-defensins contain Lys in their...
mature domain sequences. By contrast, Lys appears to be the more frequent cationic residue found in more than 30 putative human β-defensin genes (with an Arg-to-Lys ratio of ~4:6). The Arg/Lys disparity seen in mammalian α-defensins sharply contrasts the fact that Arg is one of the least frequent amino acids in proteins. In fact, the frequency of Arg (5.2%) in the genomes of living organisms (Archaea, bacteria, and eukaryotes) is on average lower than that of Lys (6.3%) despite six codons in the genetic code for Arg as opposed to only two for Lys (26). To better understand why Arg is strongly selected over Lys in human α-defensins, but not in β-defensins, and how the cationicity of defensins impacts their antimicrobial function at the atomic level, we replaced three out of four Arg residues in HNP1 by each of the following six α-amino acids: Lys, ornithine (Orn), diaminobutyric acid (Dab), diaminopropionic acid (Dap), N,N-dimethyl-Lys (diMeLys), and homo-Arg (homoArg) (Fig. 1). The N-terminal Arg5 in HNP1 was left unchanged because it forms a conserved, loop-supporting salt bridge with Glu13, contributing to α-defensin folding and stability (27). In addition, we mutated all four Lys residues to Arg in human β-defensin 1 (K→ArghBD1). Here we describe the synthesis and characterization of the six HNP1 analogs and K→ArghBD1, their bactericidal activity against Gram-positive and -negative strains, and their ability to induce leakage of an encapsulated fluorophore from large unilamellar vesicles (LUVs) composed of negatively charged phospholipids, palmitoyl-oleoyl-phosphatidylglycerol (POPG). To confirm the correct fold and describe the structural implications of the substitutions, we also determined medium-to-high resolution crystal structures of K→ArghBD1 and three HNP1 analogs (Lys, Orn, and Dab).

MATERIALS AND METHODS

Total Chemical Synthesis of HNP1 Analogs and K→ArghBD1—The amino acid sequence of HNP1 is as follows: 1ACYCRIPA-CIAGERRYGTCIYQGRRLWAFCC30. The Arg residues in boldface type were replaced by each of the following six Boc-protected α-amino acids purchased from Bachem Biosciences, Lys, Orn, Dab, Dap, diMeLys, and homoArg. Preparation of HNP1 and its synthetic analogs by Boc solid phase peptide synthesis (SPPS) was essentially as described previously (28). Briefly, all seven peptides, i.e. wild type HNP1, Lys-HNP1, Orn-HNP1, Dab-HNP1, Dap-HNP1, diMeLys-HNP1, and homoArg-HNP1, were synthesized on Boc-Cys(4MeBzl)-OCH2-PAM resin using an in-house chemistry tailored from the N,N-diisopropylethylamine in situ neutralization/2-(1H-benzo-triazol-1-yl)(3-ethyl-4-methyl-5-isoxazolyl)-1,1,3,3-tetramethyloxylumhexafluorophosphate activation protocol originally developed by Kent and co-workers for Boc SPPS (29, 30). The following side chain protections were used: Arg(tosyl), Cys(4MeBzl), Glu(OcHxl), Thr(Bzl), Tyr(BrZ), Trp(CHO), Lys(Fmoc), Orn(Fmoc), Dap(Fmoc), Dap(Fmoc), and diMeLys(Fmoc). After chain assembly and Boc/Fmoc removal, the peptides were deprotected and cleaved by anhydrous HF in the presence of 5% p-cresol at 0 °C for 1 h, followed by precipitation with cold ether. Synthesis of K→ArghBD1 (1DHYNCVSS-GQQCLYSACPIFTRIQGTCRYRGRARCRR) on Boc-(tosyl)-OCH2-PAM resin was carried out in an identical fashion using the following side chain protections: Arg(tosyl), Asn(Xan), Asp(OcHxl), Cys(4MeBzl), His(Bom), Ser(Bzl), Thr(Bzl), Tyr(BrZ). All crude peptides were purified to homogeneity by preparative C18 reversed phase (RP) HPLC on a Waters Delta Prep 600 system, and their molecular masses were ascertained by a Micromass ZQ-4000 single quadrupole electrospray ionization mass spectrometer (ESI-MS).

Oxidative folding/disulfide formation of synthetic α-defensins was carried out, as described previously, in the presence of reduced (3 mM) and oxidized (0.3 mM) glutathione, 2 mM urea, and 25% N,N-dimethylformamide, pH 8.3 (28). Folding of K→ArghBD1 was performed using a published protocol designed for wild type hBD1 and hBD2 (31). The folding reactions, monitored by analytical RP-HPLC, proceeded overnight at room temperature, and the desired products, characterized by shortened retention times and a loss of six mass units due to formation of three disulfide bonds, were purified to homogeneity by preparative RP-HPLC. To verify the correct disulfide connectivity in HNP1 analogs and in K→ArghBD1, mass mapping of peptide fragments generated by complete proteolysis with a combination of chymotrypsin and trypsin was performed. All defensins were quantified by UV absorbance measurements at 280 nm using molar extinction coefficients calculated according to the algorithm published by Pace et al. (32).

Structural Studies—Crystals of K→ArghBD1, Lys-HNP1, Orn-HNP1, and Dab-HNP1 were grown using the conditions from the commercial crystallization screens. The crystallization conditions as well as compositions of solutions used for cryo-preservation of crystals are described in supplemental Table S1. All x-ray experiments were conducted at 100 K. The x-ray data for Lys-HNP1 and Orn-HNP1 were collected using the radiation (λCu = 1.5478 Å) originated from the rotating anode mounted on the Rigaku-Ru200 generator that operated at 100 V and 50 mA and focused by the Osmic mirrors (Rigaku). The diffraction patterns were recorded using the image plate detector, MAR345db (Mar Research). The final x-ray data for the crystals of K→ArghBD1 and Dab-HNP1 were collected using the synchrotron radiation (beamline 22BM on the SER-CAT station at the Advanced Photon Source, Argonne National Laboratory), with the wavelength set to 0.96 Å. The experimental intensities were recorded using the MAR CCD300 detector (MAR-
Research). All data were processed and scaled using HKL2000 (HKL Research) (33). The basic characteristics of the crystals are shown in Table 1, and the complete data collection statistics are included in the supplemental Table S2.

The initial phases for all four derivatives were determined by the molecular replacement method with the program Phaser (34) using structures of the monomeric hBD1 (wt) and HNP1 (wt) as search models. The structures were refined with the program Refmac 5 (35) with the final values of crystallographic R-factors equal to 0.108 (K- RhBD1), 0.154 (Dab-HNP1), 0.195 (Lys-HNP1), and 0.201 (Orn-HNP1). The asymmetric units of the first two structures contain single monomers of the proteins, whereas two monomers are present in asymmetric units of the Lys and Orn derivatives. The detailed refinement statistics are included in Table 2. The final coordinates and experimental structure factors have been deposited with the Protein Data Bank.

**Antibacterial Activity Assay—Antimicrobial assays against Escherichia coli ATCC 25922 (Microbiologics) and Staphylococcus aureus ATCC 29213 (Microbiologics) were conducted using a previously published 96-well turbidimetric method dubbed virtual colony counting (36). A 2-fold dilution series of defensin, ranging from 0.5 to 125 µg/ml in 10 mM sodium phosphate, pH 7.4, was incubated at 37 °C for 2 h with E. coli or S. aureus (1 x 10^6 colony-forming units/ml), followed by addition of twice-concentrated Mueller-Hinton broth (2 x MHB) and 12-h kinetic measurements of bacterial growth at 650 nm. vLD_50 and vLD_90 values were reported as the concentration of defensin resulting in the killing of 50% and 90% of bacteria, respectively. For salt dependence experiments, bacteria were exposed to a fixed concentration of defensin at 25, 50, or 125 µg/ml for 2 h in the presence of varying concentrations of NaCl (0, 6.25, 12.5, 25, 50, and 100 mM) before addition of 2 x MHB and subsequent measurements of growth kinetics.

**TABLE 1**

| Derivative     | Space group | Unit cell parameters (Å) | Max. resolution Å |
|----------------|-------------|--------------------------|-------------------|
| K- RhBD1       | P2_1,2_1    | a = 20.37, b = 27.41, c = 55.08 | 1.36              |
| Dab-HNP1       | P3_2,1      | a = b = 38.82, c = 31.56   | 1.60              |
| Lys-HNP1       | P4_2,2      | a = b = 57.4, c = 96.97    | 2.40              |
| Orn-HNP1       | P4_2,2      | a = b = 57.4, c = 96.97    | 2.40              |

**RESULTS**

**Synthetic Access to HNP1 Analogs and K- RhBD1**

We established robust synthetic access to and efficient folding protocols for all six human α-defensins and several hBDs (28, 31, 38). Synthesis and oxidative folding of the HNP1 analogs and K- RhBD1 used in this study were essentially as described previously (28, 31). Shown in Fig. 2 are the six α-defensins, i.e. Lys-HNP1, Orn-HNP1, Dab-HNP1, Dap-HNP1, diMeLys-HNP1, and homoArg-HNP1, on analytical C18 RP-HPLC and ESI-MS; synthetic K- RhBD1 is shown in the supplemental Fig. S1. The determined molecular mass of each analog was within experimental error of the theoretical value calculated on the basis of the average isotopic compositions of folded defensins. We previously used mass mapping of peptide fragments generated by a combination of proteolytic digestion and Edman degradation to establish native disulfide connectivities in synthetic HNP1 and hBD1 (28, 31, 39). Hydrolysis of the...
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FIGURE 2. The six synthetic HNP1 analogs analyzed on RP-HPLC and ESI-MS. The chromatographic data were obtained at 40 °C on a Waters symmetry C18 column (5 µm, 4.6 × 150 mm) using a linear gradient of 5–65% B (solvent A: water plus 0.1% trifluoroacetic acid; solvent B: acetonitrile plus 0.1% trifluoroacetic acid) at a flow rate of 1 ml/min over 30 min. The mass spectrometric data were acquired on a Micromass ZQ-4000 single quadruple mass spectrometer. The mass values in parentheses are calculated based on the average isotopic compositions of folded defensins.

six HNP1 analogs by trypsin and chymotrypsin all yielded, in addition to a two-disulfide-bridged fragment containing Cys¹-Cys³-Cys⁵-Cys⁶, a common fragment of 830.8 ± 0.4 Da, which was unambiguously identified by ESI-MS to be (C²R)(YAGTC⁴Y), consistent with native disulfide connections in α-defensins. Similarly, digestion of K₃RhBD1 resulted in three major fragments, (NC⁴VSSGGQ)(SAC⁶PIF)(C⁴R) (1762.7 Da calculated, 1763.0 ± 0.3 Da found), (C²LY)(IQGTC⁴Y) (1078.4 Da calcu-
It is worth noting that as the side chain of Lys became shorter or less hydrophobic (Lys → Orn → Dab → Dap), retention of the defensins on C18 RP-HPLC progressively increased. Evidently, the more solvent-exposed the cationic charge, the less retention the defensin manifested on C18 RP-HPLC despite its increased hydrophobicity. Consistent with this observation, homoArg-HNP1, which is more hydrophobic than Arg, eluted slightly earlier than did HNP1 (data not shown). Interestingly, defensin folding became increasingly less efficient as the cationic side chain was shortened, with the folding yield for Dap-HNP1 being the lowest in the panel (data not shown). This result may be attributed to charge burial, in general, destabilizing folded peptides and proteins. Finally, as peak broadening is commonly associated with the cationicity of peptides on RP-HPLC, perhaps not surprisingly, Dap-HNP1 with least exposed cationic charges showed a much narrower chromatographic peak compared with other α-defensin analogs.

Tertiary Structures and Disulfide Topologies of HNP1 Analogs and k̅hBD1

For four derivatives included in this study (k̅hBD1, Lys-HNP1, Orn-HNP1, and Dab-HNP1), the crystal structures have been solved and refined. The selected four samples form a representative subset of all derivatives described in this study in terms of chemistry and size of the residues used for the substitutions. The primary purpose of the structural studies was to verify correct folds and to investigate potential topological changes, if any, caused by the substitutions. Each of the four derivatives forms a different type of crystal, eliminating the possibility of any bias because of specific crystal packing. When the tertiary structures of these derivatives were compared with the corresponding wild type proteins (hBD1 and HNP2), no significant structural changes were detected (Fig. 3). Other results of these comparisons are included in the Supplemental Materials. In all four cases the connectivity and stereochemistry of disulfide bridges are identical to the wild type defensins, and so are the networks of other intra-molecular interactions (hydrogen bonds, hydrophobic interactions, and salt bridges). It is thus apparent that the substitutions described for the hBD1 and HNP1 derivatives in this study did not cause obvious changes in their tertiary structures. The approximated distributions of electrostatic charges on the surfaces of the monomers of the derivatives are nearly indistinguishable from the distributions derived from the in silico-generated models based on the structures of the wild type proteins.

Oligomerization of HNP1 Analogs

Somewhat different are the results obtained from the comparison of the tertiary structures of the HNP1 derivatives (for hBD1, a monomer is believed to be its native form (40)). Most human α-defensins, previously studied by the methods of structural biology, readily form well conserved dimers (18–20),
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FIGURE 4. Survival curves of *E. coli* ATCC 25922 (left) and *S. aureus* ATCC 29213 (right) exposed to HNP1 or HNP1 analogs. Strains were exposed to the peptides at concentrations varying 2-fold from 0.5 to 125 μg/ml. Each curve is the mean of three separate experiments. Points shown below 1 × 10^−3 represent no surviving colonies.

TABLE 3
Antimicrobial activity against *E. coli* ATCC 25922 and *S. aureus* ATCC 29213

|        | E. coli | S. aureus |
|--------|---------|-----------|
| Arg    | vLD50   | vLD90     |
| Lys    | 6.4 ± 1.5 | 42.4 ± 25.2 |
| Orn    | 17.8 ± 3.6 | 33.9 ± 5.9 |
| Dab    | 3.2 ± 0.9 | 2.9 ± 0.8 |
| Dap    | 8.7 ± 4.4 | 43.7 ± 0.7 |

In which the second (central) β-strands of two monomers interact symmetrically, primarily through a network of hydrogen bonds. Virtually identical dimers are also present in the crystal structures of Lys-HNP1 and Orn-HNP1. Their topological comparison with the dimers of HNP2 is shown in the supplemental Fig. S2. In the crystal structure of Dab-HNP1, however, the central β-strand (residues 18–20) of one monomer interacts with the first β-strand (residues 2–4) of another primarily through four backbone hydrogen bonds. This asymmetric mode of dimerization results in a string of interacting monomers spanning through the crystal lattice. Analysis of the intermolecular contacts in the Dab-HNP1 crystal did not yield an obvious answer as to why the atypical dimerization pattern was observed. However, the asymmetric mode of dimerization seen in Dab-HNP1 should be disfavored for wild type human α-defensins and their derivatives with long cationic side chains at positions 14 and 24, as they would be projected toward each other.

Defensin Analogs Differentially Kill *E. coli* and *S. aureus*

The bactericidal activity of HNP1, HNP1 analogs, hBD1, and K→HBD1 was tested in a low salt medium (10 mM phosphate buffer) against the *E. coli* 25922 and *S. aureus* 29213 strains. The survival curves for α-defensins are shown in Fig. 4, and the vLD50 and vLD90 values summarized in Table 3. As expected, α-defensins, in general, showed a greater killing efficiency against *S. aureus* than against *E. coli*. Wild type HNP1, whereas exhibiting bactericidal activity against both strains comparable with the previously published results (19, 27, 36), was by far the most potent defensin in the panel. At the two highest peptide concentrations tested (62.5 and 125 μg/ml), S. aureus did not recover to exposure with HNP1, a reduction in survival by at least 6 orders of magnitude. In contrast, the killing efficiency of HNP1 against *E. coli* appeared maximal, a reduction in survival by 2 orders of magnitude at comparable peptide concentrations. Pairwise comparisons are described as follows.

Arg Versus Lys in HNP1—Replacement of arginine residues with Lys significantly reduced the toxicity of HNP1 against *S. aureus*, as evidenced by the difference in survival of at least 3-to-4 orders of magnitude at the highest peptide concentrations (62.5 and 125 μg/ml). The vLD90 value of Lys-HNP1 for *S. aureus* increased 5-fold, compared with that of wild type HNP1. By contrast, the deleterious effect of Arg → Lys mutations was less pronounced on the killing of *E. coli*, reflected by a modest 2-fold increase in vLD90. The survival of *E. coli* differed by approximately half a log in the presence of 62.5 or 125 μg/ml of HNP1 versus Lys–HNP1. Nevertheless, Arg was clearly superior to Lys in α-defensins against both Gram-positive and -negative strains of bacteria.

We incubated both defensins with *S. aureus* for different lengths of time before kinetic measurements of bacterial growth. As shown in Fig. 5, although there was little change in vLD90 for HNP1, the vLD90 value of Lys-HNP1 progressively decreased from 123 to 43.7 μg/ml as the incubation time lengthened from 15 min to 2 h, indicating that more time was
needed for the Lys mutant to exert improved bacterial killing. The finding suggests that Arg is faster acting on bacteria than Lys in HNP1.

The bactericidal activity of defensins is attenuated by salt because of weakened electrostatic interactions with anionic microbial membranes. To investigate the salt effect, we carried out functional assays at a fixed defensin concentration in the presence of varying concentrations of NaCl, from 0 to 100 mM. As shown in Fig. 6, at a peptide concentration of 125 μg/ml, complete killing of S. aureus by HNP1 was achieved at the highest salt concentration used, where much of the bactericidal activity of Lys-HNP1 was abolished. As the peptide concentration was lowered from 125 to 50 and to 25 μg/ml, while Lys-HNP1 remained ineffective against S. aureus across the entire salt concentration range, the difference in bacterial activity between HNP1 and Lys-HNP1 became less pronounced, particularly at the high salt concentrations. These results demonstrate that the greatest disparity between Arg and Lys in α-defensins against S. aureus can be achieved at low salt and high peptide concentrations.

In salt dependence experiments with E. coli, the highest peptide concentration (125 μg/ml) was used in order to obtain sufficient killing. As expected, a maximal activity against E. coli was observed for HNP1 and Lys-HNP1 without salt. However, at greater than 50 mM NaCl, both defensins lost effectiveness against the bacterium. Interestingly, compared with that of Lys-HNP1, the bactericidal activity of HNP1 was initially unaffected by low concentrations of salt (<12.5 mM) and became more sensitive in the mid-salt concentration range (from 12.5 to 50 mM). The biphasic survival curve gave rise to a salt concentration of 12.5 mM at which the greatest selectivity of Arg over Lys was observed in HNP1 against E. coli. Taken together, the results obtained from salt-dependent functional assays suggest that the bactericidal activity of HNP1 appears more salt-sensitive than that of Lys-HNP1.

Arg Versus Lys in hBD1—To compare Arg with Lys in the context of hBD1, we performed a separate antimicrobial activity assay on hBD1 and K-3hBD1 using wild type HNP1 as positive control (Fig. 7). HBD1 was significantly less efficient than HNP1 in the killing of both E. coli and S. aureus. Furthermore, as is the case for β-defensins in general, hBD1 showed modest strain selectivity toward the Gram-negative bacterium. Overall, Arg and Lys in hBD1 were found similar with respect to the killing of S. aureus and E. coli. For both strains, K-3hBD1 was marginally more effective than hBD1 in the middle concentration range. However, at the highest peptide concentrations used, the difference in killing efficiency between K-3hBD1 and hBD1 became sufficiently small. Salt dependence experiments at a fixed β-defensin concentration of 125 μg/ml were also performed on hBD1 and K-3hBD1 with both E. coli and S. aureus. Although K-3hBD1 appeared slightly more active than hBD1 in the absence of salt, the Arg-containing β-defensin showed greater salt sensitivity than wild type hBD1 (data not shown), in agreement with the earlier findings on α-defensins.

Effect of the Length of Cationic Side Chains—Lys-, Orn-, Dab-, and Dap-HNP1 comprise a homologous series of α-defensin analogs whose cationic side chains are progressively shortened by one methylene group (–CH2–). These analogs allowed us to examine the effect of the length of cationic side chains or of charge exposure on the antimicrobial activity and specificity of α-defensins. For both E. coli and S. aureus, a strong correlation was observed between the bactericidal activity of defensins and the length of cationic side chains.

The relative potency of the four defensin analogs was as follows: Lys-HNP1 > Orn-HNP1 > Dab-HNP1 > Dap-HNP1 (Fig. 4 and Table 3). At the highest peptide concentration tested, the three defensin analogs that contain noncoded amino acids Orn-, Dab-, and Dap were insufficient for 90% killing of either bacterial strain. To achieve 50% killing of S. aureus, the required peptide concentration of Dap-HNP1 was 20-fold that of Lys-HNP1, 7-fold that of Orn-HNP1, and 5-fold that of Dab-HNP1. For E. coli, the ratio of VLD50 of Dap-HNP1 to Lys-HNP1, Orn-HNP1, and Dab-HNP1 was at least 14, 3, and 2, respectively. Addition of different concentrations of NaCl in the assay medium apparently did not impact the relative potency of the four defensin analogs (Fig.
These results are indicative of the importance of the solvent accessibility of cationic charges in bacterial killing by defensins.

Arg Versus homoArg in HNP1—To test whether an elongated cationic side will enhance the antimicrobial activity of α-defensins, we incorporated into HNP1 homo-Arg, one methylene...
longer in the side chain than Arg. To our surprise, whereas homoArg-HNP1 was slightly less active than HNP1 in the killing of *S. aureus* (vLD$_{90}$ 17.4 versus 8.7 µg/ml; vLD$_{50}$ 3.0 versus 3.2 µg/ml), it showed significantly weaker toxicity toward *E. coli* compared with HNP1 (vLD$_{90}$ >125 versus 17.8 µg/ml; vLD$_{50}$ 10.4 versus 6.4 µg/ml). homoArg-HNP1 appears to be the most strain-specific defensin tested in the panel, with a strong killing selectivity for the Gram-positive bacterium.

**Lys Versus diMeLys in HNP1**—Proteins can undergo post-translational methylation on nitrogen or oxygen atoms. Methylation of the e-NH$_2$ of lysine does not alter the cationic charge but does increase hydrophobicity and steric bulk (41). We found that diMeLys-HNP1 was weaker than Lys-HNP1 against the bacteria tested. The vLD$_{90}$ and vLD$_{50}$ values of diMeLys-HNP1 increased 2-fold for both strains compared with those of Lys-HNP1. Interestingly, diMeLys-HNP1 showed greater killing efficiency against *E. coli* than against *S. aureus* at comparable peptide concentrations. The selectivity of diMeLys-HNP1 toward *E. coli* is in contrast to that of homoArg-HNP1 toward *S. aureus*.

**Defensin Analogs Differentially Induce Leakage of LUVs**

Liposomes encapsulating fluorophores are commonly used as a model system for studies of the interaction between phospholipid membranes and defensins. We measured the ability of hBD1, K-RhBD1, and all α-defensins except for homoArg-HNP1 to induce leakage of encapsulated fluorophores from LUVs composed of the negatively charged, fully unsaturated phospholipid POPG at two different salt concentrations. Several conclusions can be drawn from the dose-dependent leakage curves (Fig. 8). First, as expected, high salt inhibited the membrane activity of defensins because of weakened electrostatic interactions. Second, HNP1 and α-defensin analogs were significantly more active than the hBD1/K-RhBD1 pair. Third, in contrast to the findings from antimicrobial activity assays, the Lys → Arg mutation in hBD1 substantially reduced the membrane activity of the β-defensin.

At low salt concentration, Lys-HNP1 was marginally more active than HNP1. However, at high salt concentration, a reversal resulted. This finding suggests that Arg was more tolerant to salt than Lys, in contrast to the bactericidal activity data. The relative potency of the membrane activity of HNP1 and the four HNP1 analogs was as follows: HNP1 > Orn-HNP1 > Dap-HNP1 > Dab-HNP1, in sharp contrast to the observed correlation between the length of cationic side chains and the bactericidal activity of α-defensins. N-Methylation of Lys apparently weakened the ability of Lys-HNP1 to induce LUV leakage, particularly at high salt concentration. Overall, the membrane activity of the eight defensins tested poorly correlated with their bactericidal activity against either Gram-positive or Gram-negative bacteria.

**DISCUSSION**

Cationic AMPs are structurally amphiphilic, with clustered cationic residues and patches of apolar residues segregated across the molecule for optimal membrane association and permeabilization. It is generally accepted that the bactericidal activity of cationic AMPs is dictated by interplay between charge and hydrophobicity, of which charge is often a more prominent factor (42). Replacement of Arg in defensins or other classes of cationic AMPs by neutral or oppositely charged residues invariably leads to dramatic reduction or loss of activity in bacterial killing (43–45). Conversely, introduction of additional cationic charges often enhances bactericidal activity (46). Although the functional importance of the cationic charge in defensins is well recognized, why Arg is predominantly selected over Lys in α-defensins but not in β-defensins remains poorly understood.

King and Jukes (47) first proposed neutral evolution to interpret a strong correlation between observed frequencies of amino acids in proteins and the number of codons assigned to them in the genetic code. Several amino acids deviate from the strong correlation and are interpreted as evidence for selection (48). Arg, despite its six codons, is one of the least frequent amino acids and is strongly selected against proteins (49, 50). In fact, the disparity between expected and observed frequencies is greater for Arg than for any other amino acid.

The studies reported here afford a rare glimpse at the Arg/Lys disparity in defensins at one functional level. We found that...
Arg is a better residue than Lys in defensins with respect to their ability to kill bacteria, even though the functional difference between Arg and Lys is more evident in α-defensins than in β-defensins, and is more evident at low salt concentrations than at high salt concentrations. For α-defensins, the Arg/Lys disparity is much more pronounced with *S. aureus* than with *E. coli*; the Arg-rich HNP1 kills *S. aureus* faster than its Lys-rich analog. Naturally, the question is why?

Both known as polar residues, Arg and Lys possess side chains that are amphiphilic in nature, containing, in addition to the positively charged guanidino (pKₐ = 11–13) or amino group (pKₐ = 9–10), a significant fraction of apolar hydrocarbons. The side chain of Lys is more hydrophobic than that of a slightly bulkier Arg. In fact, estimated hydrophobic effect for side chain burial for Lys is similar to that for Val and Arg is similar to Ala (51–53). Furthermore, side chain interactions of Arg mainly involve the guanidinium group, whereas Lys has contacts with other residues through both its methylene groups and its amino group (54, 55). However, the most striking difference between Arg and Lys lies in the stronger ability of Arg to engage in electrostatic interactions such as salt bridges, H-bonds, and cationic-aromatic or cationic-π contacts (54). This enhanced capacity of Arg for electrostatic interactions appears to stem from the Arg charge delocalized over the guanidinium group versus a localized charge for Lys. As a consequence, the guanidinium group is capable of forming more extensive and stronger H-bonds (up to five) with other donors and/or acceptors, which may explain why Arg is a functionally better cationic residue than Lys in the electrostatic force-mediated killing of bacteria by defensins. It is noteworthy, however, that highly variable results have been reported for other structural classes of cationic AMPs with respect to the importance of Arg versus Lys (56–59), suggesting a context-dependent mechanism for microbial killing.

Arg residues are functionally selected over Lys in other classes of proteins such as DNA-binding proteins (60) and heparin-binding proteins (61). Increased hydrogen bonding ability of Arg is also thought to be a major reason that Arg is favored over Lys in stabilizing small proteins (62). One notable exception is hyperthermophiles that feature a greater proportion of charged residues. In the majority of hyperthermophilic genomes, the excess of positively charged residues is almost entirely due to lysines but not arginines. Berezovsky et al. (63) argued that lysines have a much greater number of accessible rotamers than equally buried arginines in folded states of proteins, thus preferentially stabilizing the native state entropically.

It is worth noting that eosinophil cationic protein, which resembles HNP1 with respect to microbial killing (64), represents an additional instance of positive Darwinian selection in an antimicrobial peptide (65). The eosinophil cationic protein gene belonging to the ribonuclease gene family had a significantly higher rate of nonsynonymous nucleotide substitution during the early stage of evolution. As a result, the number of Arg residues increased substantially in a short period of evolutionary time after gene duplication, producing the antimicrobial function of eosinophil cationic protein. Not surprisingly, neutrophil granule-associated, bactericidal serine proteases such as elastase, cathepsin G, and azurocidin share an extremely large Arg/Lys disparity in their sequences. For instance, human neutrophil elastase contains 22 Arg residues and no Lys; the ratio of the total number of Arg residues to Lys in these three antimicrobial proteins is an astonishing 78:5. However, the selective pressure for Arg over Lys in these bactericidal serine proteases, which are important for phagocytosis, is cell/tissue-specific. In fact, the Arg/Lys ratios in many other homologous serine proteases such as granzyme B in NK cells and T-lymphocytes and pancreatic trypsin and chymotrypsin are quite normal.

In the case of α-defensins, one could argue that functional conservation (bacterial killing) at physiological salt concentrations may be the selective pressure for Arg over Lys. However, this argument clearly lacks merit for β-defensins where Lys is the more frequent cationic residue than Arg, and the functions of the bulk of more than 30 putative human β-defensin genes remain largely unknown. To date, all human defensins studied possess, in addition to antimicrobial properties, other biological activities, and many functions of these important immunological molecules have yet to be elucidated. β-Defensins chemotact various blood cells to the site of infection, stimulate cell differentiation, and participate in sperm maturation (66–72). Even for the better characterized human α-defensins, their functions, in addition to bacterial killing through membrane disruption, have been shown to be pleiotropic and multifaceted in innate and adaptive immunity, reflected by their “promiscuous” interactions with cellular receptors, carbohydrates, and glycoproteins, bacterial toxins and cell wall components, nucleic acids, etc. One possible explanation may be that human α-defensins play a more prominent role in direct microbial killing, whereas β-defensins act primarily as immune effector molecules. Activities of α-defensins other than microbial killing could be either a “by-product” of their molecular composition or a result of lesser evolutionary pressures. In the case of β-defensins, however, evolution toward becoming immune effectors may have required more flexibility in the selection of cationic residues.

In a powerful example, facile synthetic access to α-defensins via SPPS enabled noncoded amino acids to be readily incorporated into the sequence, substantially improving our ability to decipher the molecular basis for defensin function. Use of the homologous series of noncoded amino acids allowed us to examine the effects of charge exposure on the antimicrobial activity and specificity of α-defensins. To verify the correct defensin fold and native disulfide connectivity, we determined the medium-to-high resolution crystal structures for four derivatives. The results show that the modifications introduced to the defensin molecules did not alter in any significant way the topological properties of resulting proteins. The two coded cationic residues, Arg and Lys, appeared to have optimal chain lengths for their bactericidal function, as shortening Lys or lengthening Arg in HNP1 invariably caused deleterious effects on bacterial killing. Shown in Fig. 9 is the distribution of charged atoms and of the electrostatic potential on the solvent-accessible surface of HNP1 and its five analogs. Analysis of this figure allows for several observations. First, the modifications of basic residues slightly alter the shape of the molecular envelope.
Second, changing the length of the side chains in basic residues does not affect significantly the electrostatic potential projected on the molecular surface (Fig. 9B). Consequently, there exists no correlation between electrostatic potential distributions and bactericidal activities of different HNP1 analogs. Third, when the solvent-accessible surface (or molecular surface) is colored according to the net charge of the contributing atom (Fig. 9A), the screening effect of the atoms adjacent to the charge centers becomes quite clear. This effect is well exemplified by diMe-Lys-HNP1, where the methylated nitrogen atoms in residues 14, 15,
and 24 are nearly completely buried in the molecular envelope. A similar yet somewhat less pronounced effect can be seen for other analogs. Therefore, it appears that changes in the exposure of charged atoms correlate reasonably well with the changes of bactericidal properties. Finally, the elongated charged side chains in homoArg-HNP1 ensure, on the one hand, exposure of positively charged atoms to the bacterial membrane, but they may hinder, on the other hand, proximal interactions involving noncharged residues of HNP1.

Finally, we found that the membrane activity of the eight defensins tested poorly correlated with their bactericidal activity against either Gram-positive or Gram-negative bacteria. Poor correlation was previously noted for all six human α-defensins using LUVs of various compositions and other classes of AMPs (73–75), suggesting that LUVs as a frequently used model membrane system are inadequate for an accurate depiction of the antibacterial activity and specificity of defensins because of mechanistic complexity of microbial killing by cationic antimicrobial peptides. Better model membrane systems are needed to mimic the interplay between charge and hydrophobicity of defensins on one hand and between lipid composition and cell wall/membrane components on the other hand.

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