Mammalian α-defensins, expressed primarily in leukocytes and epithelia, kill a broad range of microbes, constituting one of the first lines of innate immune defense against infection. Nine amino acid residues, including six cysteines, one glycine, and a pair of oppositely charged residues Arg/Glu, are conserved in the otherwise diverse sequences of all known mammalian α-defensins. Structural analysis indicates that the two charged residues form a salt bridge, likely stabilizing a protruding loop in the molecule. To investigate the structural and functional roles of the conserved Arg5–Glu13 salt bridge in α-defensins, we chemically prepared human neutrophil α-defensin-2 (HNP2) and five HNP2 analogs, R5E/E13R, E13Q, E13R, R5T/E13Y, and R14A. In contrast to HNP2 and R14A–HNP2, none of the four salt bridge analogs was capable of folding into a native conformation in the context of isolated defensin domains. However, when covalently attached to the 45-residue pro-HNP2 propeptide, the salt bridge analogs of HNP2 in their pro-forms all folded productively, suggesting that the Arg5–Glu13 salt bridge is not required for correct pro-α-defensin folding. When assayed against both *Escherichia coli* and *Staphylococcus aureus*, the six α-defensins showed bactericidal activity that correlated with the number of net positive charges carried by individual molecules in the panel, irrespective of whether or not the Arg5–Glu13 salt bridge was decimated, suggesting that Arg5 and Glu13 are not functionally conserved. Proteolytic resistance analysis with human neutrophil elastase, one major protease contained in azurophils with HNPs, revealed that destabilization of the salt bridge dramatically accelerated defensin degradation by the enzyme. Thus, we propose that the Arg5–Glu13 salt bridge found in most mammalian α-defensins is conserved for defensin *in vivo* stability.

Defensins are a family of cationic antimicrobial mini-proteins expressed predominantly in leukocytes and epithelial cells. They directly inactivate a broad range of microbes presumably through disruption of the anionic microbial membrane, constituting an effective arsenal in the innate immune defense against microbial infection (1–4). Defensins also function as effector molecules in adaptive immunity by chemoattracting and activating certain types of immune cells via receptor-mediated signaling pathways (5–9). In humans, defensins are classified into α-defensins, expressed predominantly in leukocytes and epithelial cells. They directly inactivate a broad range of microbes presumably through disruption of the azurophilic granules of neutrophils. HNP1–3, differing by a single amino acid residue at the N terminus, collectively represent the most abundant cellular protein in neutrophils. Three additional members of the human α-defensin family were subsequently identified, including HNP4 from neutrophils and HD5–6 from small intestinal Paneth cells (12–16). Despite the difference in tissue distribution, all human α-defensins are synthesized *in vivo* as inactive precursor proteins that require proteolytic excision of their N-terminal inhibitory anionic propeptide for maturation. Whereas HNPs are stored in azurophil granules as an active, mature form and kill engulfed microbes during phagocytosis, HD5–6 are stored as inactive prodefensins in granules, and proteolytically activated and secreted into the gut lumen in response to bacterial stimulation. Ganz and colleagues (17–19) demonstrated in *in vivo* studies of pro-HNP1 that the 45-residue propeptide is important for correct defensin subcellular sorting and trafficking.

Despite the early discovery of these immunological molecules and a growing recognition of their multifaceted functional roles in innate and adaptive immunity, the sequence rules and structural determinants dictating the antimicrobial activity and specificity of human α-defensins have not yet been established and remain poorly understood. Sequence alignment analysis shows that α-defensins from human, mouse, rhesus macaque, rabbit, rat, and guinea pig share ~30% sequence identity. Among the totally conserved amino acid residues are six cysteines and one invariant Gly17 (HNP1 numbering, see Fig. 1). In addition, there exists a pair of highly conserved charged residues, Arg5 and Glu13, forming a salt bridge as indicated by structural analysis of various α-defensin molecules. One notable exception is guinea pig neutrophil α-defensins, where the corresponding residues are Thr8 and Tyr13 (20). An obvious question is: why are certain amino acid residues conserved in the otherwise diverse sequences of all known mammalian α-defensins?

The six invariant Cys residues in α-defensins form three intramolecular disulfide bonds, i.e. Cys1–Cys6, Cys2–Cys4, and Cys3–Cys5, thus indispensable for defensin folding and structural stabilization. We have recently shown that the invariant Gly17, located in an atypical, classic-type β-bulge with the backbone torsion angles (ϕ, ψ) disallowed for L-amino acids but permissible for D-enantiomers, constitutes a signature structural motif (GXC) essential for correct defensin folding (21). The structural as well as functional roles of the remaining conserved element in α-defensins, i.e. the Arg5–Glu13 salt bridge, has yet to be elucidated. This report describes characterizations of HNP2 and its four salt bridge analogs, i.e. R5E/E13R, E13Q, E13R, and R5T/E13Y, and of a control analog R14A–HNP2. Our findings shed light on the molecular basis of the conservation of Arg5 and Glu13 in mammalian α-defensins.

**MATERIALS AND METHODS**

**Peptide Synthesis and Native Chemical Ligation—**Solid phase peptide synthesis of wild type human α-defensins 1–6 was previously described (22–24). For the five HNP2 analogs, R5E/E13R, E13Q, E13R, R5T/E13Y, and R14A, stepwise chain assembly was carried out on Boc-Cys(4-Me-
Bzl)-OCH$_2$-phenylacetamidomethyl resin on an ABI 433A peptide synthesizer using an in-house Boc chemistry tailored from the HBTU activator/DIEA/1-H9251/N,N-dimethylformamide (v/v) buffer to pH 8.2. For pro-HNP2 analogs, the peptides were each dissolved in a concentration of 2 mg/ml in 8M urea containing 12 mM reduced and 1.2 mM oxidized glutathione, followed by a 4-fold dilution with 0.25M NaHCO$_3$. The air-tight folding reactions proceeded overnight with gentle stirring at room temperature. All folded products were purified by RP-HPLC to homogeneity, and their molecular masses ascertained by ESI-MS.

Chemical release of the four salt bridge analogs of HNP2 from folded prodefensins was achieved by quantitative CNBr cleavage of the only Met-Cys peptide bond connecting the propeptide and the defensin domain. The reactions were carried out at a protein concentration of 2.5 mg/ml in 2.5% trifluoroacetic acid containing 25 mg/ml CNBr. Released HNP2 analogs were further purified by RP-HPLC and subsequently lyophilized. The molecular masses of the four HNP2 analogs were ascertained by ESI-MS, and, their disulfide connectivity was independently verified by mass mapping of peptide fragments generated by proteolytic digestion as well as Edman degradation according to the published procedures (24, 28). All defensin analogs were quantified by UV absorbance measurements at 280 nm using molar extinction coefficients calculated according to the algorithm published by Pace et al. (29).

**Antibacterial Activity Assay**—Antimicrobial assays against Escherichia coli ATCC 25922 (Microbiologics) and Staphylococcus aureus ATCC 29213 (BD Biosciences) were conducted using a previously detailed 96-well turbidimetric method dubbed virtual colony counting (21, 30). A 2-fold dilution series of defensin, ranging from 0.5 to 128 µg/ml in 10 mM sodium phosphate, pH 7.4, was used in the dose-dependent survival assay. For salt-dependence experiments, bacteria were exposed to a fixed concentration of defensin at 50 µg/ml for 2 h in the presence of varying concentrations of NaCl (from 5 to 150 mM) before addition of twice-concentrated Mueller-Hinton broth and subsequent measurements of growth kinetics.

**Proteolytic Resistance Assay**—Defensins at 0.5 mg/ml each were incubated at room temperature with human sputum neutrophil elastase (Elastin Products, Inc.) at 0.5 mg/ml in 50 mM Tris/HCl buffer containing 20 mM CaCl$_2$ and 0.001% Triton X-100, pH 8.3. Aliquots were withdrawn at different time intervals (0, 2, 4, 6, 8, and 24 h) and injected for
analysis on RP-HPLC. Percent residual defensin was calculated based on the ratio of the integrated peak areas of an intact molecule at a given time versus at time 0. ESI-MS was used to identify some cleavage products separated by analytical HPLC.

Elastase Inhibition Assays—Elastase, prepared as stock at 0.1 mg/ml in 50 mM NaOAc containing 20 mM CaCl₂, pH 5.0, was stoichiometrically titrated to a 96-well plate by a previously characterized synthetic protein inhibitor, turkey ovomucoid third domain (OMTKY3) (31). Specifically, the enzyme was freshly diluted to 10 μg/ml, in a total volume of 196 μl, and incubated for 5 min at room temperature with varying concentrations of OMTKY3 in 50 mM Tris/HCl buffer containing 20 mM CaCl₂ and 0.001% Triton X-100, pH 8.3. Upon addition of 4 μl of the elastase substrate Suc-Ala-Ala-Ala-p-nitroanilide at 10 mg/ml in Me₂SO, the enzyme activity, characterized by an increased UV absorbance at 405 nm resulting directly from substrate hydrolysis catalyzed by free elastase in the solution, was continuously monitored spectrophotometrically (OD collected every 6 s for 2 min) on a Amersham Biosciences V_max microplate reader. Percent residual enzyme activity was defined based on the ratio of the initial reaction rates (ΔAbs/s) obtained from time-dependent hydrolysis kinetics in the presence versus absence of OMTKY3. Potential inhibition by HNP2 at different molar concentrations against a concentration-standardized enzyme was evaluated under identical conditions except that HNP2 in up to 100-fold excess was used.

RESULTS

The Salt Bridge Is Important for the Folding of Isolated α-Defensin Domains—Reduced α-defensins are extremely prone to aggregation in solution. We have previously developed a folding protocol that uses 25% N,N-dimethylformamide and 2 M urea in the presence of a reduced/oxidized thiol pair, achieving highly efficient folding of HNP1–3 (23). Because of the small size, α-defensins contain little hydrophobic packing, and are stabilized primarily by three intramolecular disulfide bridges. This unique structural feature renders α-defensins resistant to solvent denaturation, an inherent characteristic of mini-proteins with a small change in surface area upon exposure to high concentrations of chemical denaturants (32). Thus, the key to effective oxidative folding of HNP1–3 is the use of a “sufficiently denaturing or highly solubilizing” solvent condition to eliminate peptide aggregation, thereby ensuring productive thiol-disulfide exchanges in the folding process. This “folding under denaturing conditions” approach worked exceptionally well for HNPs and a great number of α-defensin analogs (21, 33), including R14A–HNP2.

However, the four salt bridge analogs of HNP2 all failed to fold into a native conformation using the same approach, characterized by massive peptide precipitation and a highly heterogeneous chromatographic trace of the supernatant with no distinct peaks of meaningful signal intensity. Shown in Fig. 2 is a representative folding reaction of [R5E,E13R]-HNP2 monitored by analytical RP-HPLC. Various attempts to modify the solvent composition of the folding buffer met with little success (data not shown). These results suggest that the Arg⁵–Glu¹³ salt bridge is important for the folding of reduced α-defensin domains.

The Salt Bridge Is Dispensable for the Folding of Pro-α-defensins—α-Defensins are synthesized in vivo as inactive pro-forms that require proteolytic excision of the propeptide for maturation. In contrast to reduced defensin domains, denatured pro-α-defensins are highly soluble in aqueous solution and can be readily folded using mild solvent conditions (24). The 45-residue anionic propeptide is known to specifically interact, either in trans or in cis, with cationic HNP1–3. Such an interaction is believed to be of electrostatic nature and contributes to the inability of pro-HNPs to kill bacteria and to induce leakage from liposomes (19, 24). Recently, we have shown that the propeptide chaperones the folding of pro-HNPs through two independent mechanisms: solubilization of and interaction with the C-terminal defensin domain. To investigate whether mutations at Arg⁵ and/or Glu¹³ have any impact on the folding of pro-α-defensins, we prepared the four salt bridge analogs in the form of pro-HNP2 using native chemical ligation (26, 27), and studied their oxidative folding kinetics.

As was the case with the previously reported wild type pro-HNP1, the four pro-α-defensin analogs all folded productively with no apparent aggregation. Shown in Fig. 2 is the folding reaction of [R5E,E13R]-HNP2 in its pro-form at 20 h monitored on RP-HPLC. ESI-MS analysis of the folded product showed a mass decrease by 6 units compared with the expected value of 8161.4 Da calculated based on the average isoform compositions of the protein. Note that the folded pro-α-defensin, despite its relatively broad and tailing peak on HPLC, characterized by certain cationic peptides and proteins interacting with the reversed-phase column matrix, is chromatographically pure.

\[ \text{Conserved Salt Bridge in } \alpha\text{-Defensins} \]

\[ \text{[R5E, E13R]-HNP2 before folding} \]

\[ \text{UV Absorbance at 240 nm (AU)} \]

\[ \text{Retention Time (min)} \]

\[ \text{[R5E, E13R]-HNP2 after folding} \]

\[ \text{UV Absorbance at 240 nm (AU)} \]

\[ \text{Retention Time (min)} \]

\[ \text{FIGURE 2. Oxidative folding of R5E/E13R-HNP2 in the absence (left) or presence (right) of a covalently linked propeptide monitored on RP-HPLC. R5E/E13R-HNP2 was folded overnight at 0.25 mg/ml in 25% N,N-dimethylformamide and its corresponding pro-α-defensin analog at 0.5 mg/ml in 50 mM NaHCO₃, both containing 2M urea, 3 M reduced, and 0.3 M oxidized glutathione, pH 8.2. The chromatographic data were collected at 40 °C on a Waters Symmetry 300 C18 column (4.6 × 150 mm, 5 μm) running a 30-min linear gradient of 5–65% B at a flow rate of 1 ml/min. ESI-MS analysis of the folded pro-α-defensin gave an observed mass of 8161.6 ± 0.2 Da, in agreement with the expected value of 8161.4 Da calculated based on the average isoform compositions of the protein. Note that the folded pro-α-defensin, despite its relatively broad and tailing peak on HPLC, characterized by certain cationic peptides and proteins interacting with the reversed-phase column matrix, is chromatographically pure.} \]
three disulfide bridges during the folding. Similar results were also obtained with other pro-α-defensin analogs (data not shown). Folded pro-α-defensins, after HPLC purification, were subjected to CNBr cleavage of the Met-Cys peptide bond connecting the propeptide and the C-terminal defensin domain, yielding R5E/E13R-, E13Q-, E13R-, and R5T/E13Y-HNP2 (Fig. 3). The determined molecular masses of these CNBr-released HNP2 analogs are in agreement with the expected values calculated based on their average isotopic compositions (Fig. 3). Peptide mass mapping coupled with complete tryptic/chymotryptic digestion and Edman degradation confirmed the correct disulfide connectivity in these salt bridge analogs of HNP2, i.e. Cys1–Cys6, Cys2–Cys4, and Cys3–Cys5 (data not shown). Our results suggest that the Arg5–Glu13 salt bridge in the context of pro-α-defensins is not required for productive and correct protein folding.

The Salt Bridge Is Not Conserved for Antibacterial Activity—To investigate the effect of the Arg5–Glu13 salt bridge on defensin function, we compared the bactericidal activities of all six defensins in the panel against *S. aureus* and *E. coli* (Fig. 4). Consistent with our previous observations (21, 30, 33), the dose-dependent survival curves for *S. aureus* are concave-down on log-log scale, suggesting simple exponential killing and the absence of any resistance mechanism. Whereas complete killing was achieved for HNP2, E13R-HNP2, and E13Q-HNP2 against *S. aureus* (Fig. 4), consistent with our previous observations, the dose-dependent survival curves for *E. coli* and *S. aureus* are concave-up on log-log scale. The determined lethal doses (LD50, LD90, and LD99) values of R14A-HNP2 increased uniformly by 6-fold for *E. coli* and by 2-, 3-, and at least 4-fold, respectively, for *S. aureus*. By contrast, the charge-reversing mutation E13R yielded the most potent defensin in the panel, E13R-HNP2 killed bacteria significantly more effectively than wild type HNP2, as evidenced by a roughly 3-fold decrease in vLD99. These results demonstrated the functional importance of cationic residues in defensins. Similarly, removal of a negative charge, i.e. E13Q, marginally enhanced the bactericidal activity of HNP2, showing a decrease in vLD99 by 2-fold for *E. coli* and in vLD99 by 3-fold for *S. aureus*. The two double mutants, R5T/E13Y and R5E/E13R, and wild type HNP2, in which the net positive charge remained unchanged, showed comparable bactericidal activity against *E. coli*. All four sets of vLD values of these three defensins varied within a factor of 2 for *E. coli*. For *S. aureus*, the difference widened in the high concentration range, where a consensus order of the relative bactericidal activity emerged as HNP2 > R5T/E13Y ~ R5E/E13R.

The bactericidal activity of defensins is attenuated by salt because of a weakened defensin-microbe interaction mediated largely by electrostatic forces. To investigate the effect of salt on the activity of HNP2 and its analogs, we carried out a functional assay at a fixed defensin concentration of 50 μg/ml in the presence of varying concentrations of NaCl, from 5 to 150 mM. As shown in Fig. 5, the bactericidal activity progres-
sively decreased in a dose-dependent manner as the NaCl concentration increased. Furthermore, the survival of \textit{E. coli} was noticeably more sensitive to the change in salt concentration than that of \textit{S. aureus}. Two important conclusions can be drawn. First, the relative potency of the bactericidal activity of HNP2 and its analogs appeared independent of the salt concentration. A consensus order for both strains appeared to be: E13R (\textit{HNP2}) > R5E/E13Y > E13Q > E13R > R5T/E13Y > R14A, which correlated with the number of net positive charges shown in parentheses. Second, as was the case with bactericidal activity, salt resistance of defensins was influenced less by whether or not the conserved Arg\textsuperscript{5}–Glu\textsuperscript{13} salt bridge is decimated or weakened, but more by the number of net positive charges defensins carry. For example, in the case of \textit{S. aureus}, the bactericidal activity of E13R-HNP2 was more salt-resistant than that of wild type HNP2. Taken together, these data suggest that the Arg\textsuperscript{5}–Glu\textsuperscript{13} salt bridge in \(\alpha\)-defensins is not conserved functionally.

The Salt Bridge Is Important for the Resistance of Defensins to Proteolysis by Elastase

- HNPs are stored in azurophils with several proteases including neutrophil elastase. To investigate whether the conserved salt bridge in \(\alpha\)-defensins is important for proteolytic resistance, we incu-

![FIGURE 4](image4.png)

**TABLE ONE**

| Defensin | \(vLD_{50}\) \(\mu g/ml\) | \(vLD_{90}\) \(\mu g/ml\) | \(vLD_{99}\) \(\mu g/ml\) | \(vLD_{99.9}\) \(\mu g/ml\) | \(vLD_{50}\) \(\mu g/ml\) | \(vLD_{90}\) \(\mu g/ml\) | \(vLD_{99}\) \(\mu g/ml\) | \(vLD_{99.9}\) \(\mu g/ml\) |
|----------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|
| HNP2     | 4.3 ± 1.0       | 10.0 ± 1.3      | 21.5 ± 3.4      | 87.0 ± 17.8     | 2.4 ± 0.7       | 11.5 ± 3.7      | 30.6 ± 2.7      | 59.5 ± 0.5      |
| R5E/E13R | 8.0 ± 2.8       | 17.0 ± 3.8      | 29.4 ± 1.8      | 54.4 ± 5.3      | 3.0 ± 0.1       | 24.6 ± 3.3      | 66.6 ± 3.6      | >128            |
| E13Q     | 6.8 ± 2.7       | 11.0 ± 3.8      | 19.2 ± 5.8      | 45.4 ± 11.2     | 4.0 ± 0.2       | 8.8 ± 3.1       | 11.4 ± 4.4      | 54.8 ± 3.1      |
| E13R     | 2.5 ± 0.9       | 6.9 ± 0.3       | 19.3 ± 9.3      | 30.7 ± 0.9      | 4.4 ± 0.3       | 6.5 ± 0.3       | 14.9 ± 0.0      | 24.5 ± 1.1      |
| R5T/E13Y | 5.0 ± 0.2       | 8.7 ± 1.0       | 15.5 ± 0.3      | 72.6 ± 14.3     | 2.9 ± 0.2       | 12.6 ± 3.6      | 21.4 ± 1.8      | 101 ± 13.6      |
| R14A     | 24.0 ± 0.8      | 59.8 ± 2.8      | 127             | >128            | 4.7 ± 1.4       | 37.6 ± 4.7      | >128            | >128            |

![FIGURE 5](image5.png)
bated wild type HNP2 and the salt bridge analogs of HNP2 with elastase, and monitored the breakdown of the defensins on RP-HPLC. Shown in Fig. 6 are representative HPLC traces of HNP2, R5T/E13T-, and E13Q-HNP2 before and after incubation with elastase for 8 h, and time-dependent defensin degradation kinetics over a period of 24 h. Wild type HNP2 was markedly more resistant to proteolysis than any other defensin in the panel. Within 8 h of incubation, more than 70% of E13Q-HNP2 was hydrolyzed by the enzyme, whereas 80% of wild type HNP2 remained intact. Degradation of other salt bridge analogs was equally efficient as compared with that of E13Q-HNP2, except for R5T/E13T-HNP2, in which case, an 8-h incubation with elastase resulted in a 50% loss of the defensin. ESI-MS analysis of fragment peaks identified cleavage sites consistent with the known specificity of elastase, i.e., a preference for small and/or \( \beta \)-branched aliphatic side chains at the P1 position (34, 35).

**DISCUSSION**

\( \alpha \)-Defensins adopt a three-stranded \( \beta \)-sheet core structure stabilized by three intramolecular disulfide bridges (21, 37). Aside from six invariant Cys residues, Gly\(^{17} \), Arg\(^{5} \), and Glu\(^{13} \) (HNP1 numbering) are the only conserved elements in the otherwise highly diverse sequences of mammalian \( \alpha \)-defensins. Whereas the structural and functional roles of S-S bonding and of Gly\(^{17} \) have recently been elucidated in the context of mouse \( \alpha \)-defensins (38) and HNPs (21), respectively, the importance of the conserved Arg\(^{5} \)–Glu\(^{13} \) pair has yet to be established. In all known \( \alpha \)-defensin structures, Arg\(^{5} \) and Glu\(^{13} \) form a salt bridge, strong electrostatic and H-bonding interactions between two oppositely charged residues. Shown in Fig. 8 is the Arg\(^{5} \) and Glu\(^{13} \) salt bridge found in the crystal structure of D-Ala\(^{17} \)-HNP2 (21), where two well defined H-bonds, at an average distance of 2.9 Å, are depicted between N\(^{\alpha} \) and N\(^{\alpha} \) of Arg\(^{5} \) and O\(^{\beta} \) and O\(^{\beta} \) of Glu\(^{13} \). The charge-charge interaction, along with a perpendicular disulfide bond (Cys\(^{9} \)–Cys\(^{29} \)), stabilizes the extruding loop of HNP2 encompassing Arg\(^{3} \)-Ile\(^{6} \)-Pro\(^{7} \)-Ala\(^{8} \)-Cys\(^{9} \)-Ile\(^{11} \)-Ala\(^{11} \)-Gly\(^{12} \)-Glu\(^{13} \).

To address the question of why the Arg\(^{5} \)–Glu\(^{13} \) salt bridge is conserved in \( \alpha \)-defensins, we constructed the following four HNP2 analogs:
Conserved Salt Bridge in α-Defensins

R5/E13R, E13Q, E13R, and R5T/E13Y. The two single mutations E13Q and E13R effectively eliminated the charge-charge interaction in HNP2. Whereas the charge-reversing mutations, R5/E13R, presumably maintained the salt bridge in a reversed order, R5T/E13Y likely weakened the interaction by preserving one potential side chain–side chain H-bond of Thr5 and Oγ of Tyr13 found only in guinea pig neutrophil α-defensins (20). However, unlike R14A-HNP2 and wild type HNP2, none of the four salt bridge analogs was capable of folding into a native conformation productively, suggesting that the Arg5–Glu13 salt bridge participates in an energetically critical interaction in the folding pathway of denatured α-defensin domains. Given that the loop is structurally stabilized by the Arg5–Glu13 interaction and by the Cys9–Cys29 disulfide, it may be tempting to assume that formation of the salt bridge in folding intermediates necessitates correct pairing of Cys9 and Cys29.

It is perhaps not surprising to see that the reversed “salt bridge” R5/E13R failed to rescue HNP2 from misfolding and aggregating. This may be a case where a reversed salt bridge is energetically un-equivalent to its parent one as the charged residues involved are situated in a rather different structural environment upon position switching. The Thr5–Tyr13 substitutions did not fare any better possibly because of the following two reasons. First, the potential side chain H-bonding between Thr5 and Tyr13 may be weaker than the Arg5–Glu13 salt bridge. Second, in the context of HNP2, Thr5 and Tyr13 may not be able to correctly orient their side chains to form a geometrically optimal H-bond, as a Pro12 residue immediately preceding Tyr13 in guinea pig neutrophil α-defensins is conspicuously missing in any other mammalian α-defensins.

Because α-defensins are synthesized in vivo as precursor proteins, the ultimate test of the effect of the salt bridge on defensin foldability should come from studies of pro-α-defensin analogs. Our findings showed that all four salt bridge analogs of pro-HNP2 folded correctly and productively, suggesting a diminished role of the Arg5–Glu13 salt bridge in the folding of pro-α-defensins. This is in stark contrast to the importance of the salt bridge in the folding of isolated defensin domains. Ample evidence demonstrates that the anionic propeptide specifically interacts, presumably through electrostatic forces, with neutrophil α-defensins, either intramolecularly or intermolecularly. The neutralization of cationic charges on defensins by the propeptide is thought to be the major contributing factor in the loss of the antimicrobial activity of pro-HNPs, and in the dose-dependent inhibition of HNPs by an unlinked propeptide (19, 24). We have recently shown that the propeptide catalyzes the folding of pro-HNPs by two independent mechanisms: solubilizing the aggregation-prone C-terminal domain in the reduced form, and, interacting with native-like intermediates, thus lowering the free energy of their transition states in the folding pathway. It is conceivable that the Arg5–Glu13 salt bridge is not critically involved in the interactions between the propeptide and the defensin domain as mutations targeted to eliminate or weaken the conserved salt bridge did not derail a productive folding path of pro-HNP2 analogs.

The question is: if the salt bridge is not structurally required for defensin folding, is it conserved for antibacterial activity? Our results from functional assays provided a compelling answer: no. The bactericidal activity had little to do with whether or not the salt bridge was broken or weakened. Rather, it was determined by the number of net positive charges carried by individual defensins. The finding that net positive charge dictates defensin function is not surprising. Defensins, like most cationic antimicrobial peptides, are believed to directly kill bacteria by permeabilizing the negatively charged cytoplasmic membrane, thus inducing leakage of intracellular contents (39–43). Electrostatic forces that mediate self-promoted uptake of defensins across the
Conserved Salt Bridge in α-Defensins

anionic cell wall structure confer additional selectivity of cationic defensins toward bacteria (44). Consistent with this mode of action, various studies have shown that deletion or addition of positively charged residues such as Arg dramatically alters antimicrobial activity and specificity (21, 45–47), underscoring the functional importance of net charge in microbial killing by defensins. Notably, although a correlation between structural rigidity (stability) and salt-dependent activity was reported for some antimicrobial peptides (48–51), we did not find bacterial killing by HNP2 more resistant to NaCl than by its salt bridge analogs. In contrast, our data appeared consistent with the observation that the number of positive charges in defensins correlates with antimicrobial activity at high salt concentrations (52, 53), presumably because more charged residues allow a defensin molecule to overcome high-salt inhibition of the binding interaction with negatively charged membrane lipids.

We propose that the Arg5–Glu13 salt bridge is conserved in α-defensins for in vivo stability. Several lines of reasoning support our hypothesis. First, human neutrophil α-defensins are stored in azurophilic granules together with several abundant proteases such as elastase, cathepsin G, and proteinase 3 (54). Proteolytic stability is thus an obvious prerequisite for the functioning of HNPs during phagocytosis. Second, loop regions in proteins are preferred sites targeted by proteases because of conformational adaptability required for active-site binding. Therefore, structural stabilization in the loop region is critical for averting productive proteolytic cleavage. We showed that modification of a salt bridge supporting the inhibitory reactive-site loop of the protease inhibitor eglin c caused a loss of conformational rigidity and effectively converted a potent protease inhibitor into an efficient substrate (55). Third, neutrophil elastase is known to preferably cleave peptide bonds containing Gly, Ala, Val, and Ile at the P1 position (34). The loop region in HNP1–3, i.e. Arg5–Ile6–Pro7–Ala8–Cys9–Ile10–Ala11–Gly12–Glu13, is rich in Ala and Ile residues, thus an ideal site targeted by elastase. Our findings from studies of in vitro stability of HNP2 and its salt bridge analogs are entirely consistent with the premise that Arg5 and Glu13 are conserved in α-defensins to prevent proteolytic degradation. It is worth pointing out that azurophil contains an abundant matrix composed of strongly negatively charged sulfated proteoglycans that binds to both defensins and neutrophil proteases (54). This sequestration of a salt bridge supporting the inhibitory reactive-site loop of the protease inhibitor eglin c caused a loss of conformational rigidity and effectively converted a potent protease inhibitor into an efficient substrate (55). Third, neutrophil elastase is known to preferably cleave peptide bonds containing Gly, Ala, Val, and Ile at the P1 position (34).

We recognize that the amino acid sequences in the loop region of α-defensins are highly variable, often containing multiple Arg and bulky hydrophobic residues such as Phe in neutrophil α-defensins from different species. The loop region of the much less abundant HNP4, i.e. Arg2–Leu4–Val5–Phe6–Cys7–Arg8–Arg9–Thr10–Glu11, exemplifies such variations, which are, in fact, consistent with the coexistence of other proteolytic enzymes of different specificities in azurophilic. Cathepsin G, for example, is known to preferably hydrolyze peptide bonds containing Arg/Lys and bulky hydrophobic residues such as Phe and Tyr at the P1 position (56). It is therefore conceivable that destabilization of the conserved Arg5–Glu13 salt bridge in HNP4 may lead to defensin degradation by cathepsin G and possibly by elastase as well. Notably, some α-defensins such as HD5–6 are stored as pro-forms in granules, inducibly activated by proteases such as trypsin, and secreted into the gut lumen in response to microbial stimuli (57). It is plausible that the conserved salt bridge affords these α-defensins proteolytic resistance to their processing enzymes. In fact, Ouellette and colleagues (56) demonstrated that structural stability afforded by disulfide bonding in mouse α-defensins (cryptdins), whereas not required for their antimicrobial function as shown in several β-defensins (28, 58, 59), is essential for preventing in vivo proteolytic degradation by their processing enzyme, the metalloproteinase matrilysin (38, 60). Our work has established the importance of two highly conserved, oppositely charged residues in maintaining α-defensin in vivo stability and thus biological functions of these multifaceted immunological molecules.

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