Synthesis, Structure, and Activities of an Oral Mucosal α-Defensin from Rhesus Macaque*

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The oral cavity is an environment challenged by a large variety of pathogens. Consequently, the antimicrobial peptides expressed in that environment are interesting as they evolved to defend against a broad spectrum of bacteria and fungi. Here we report the discovery of new α-defensins from rhesus macaque oral mucosa and determine the first α-defensin structure from that species. The new peptides were identified by sequencing of reverse transcriptase-PCR products obtained from oral mucosal tissues, disclosing three mucosal α-defensins, termed rhesus macaque oral α-defensins (ROADs). The peptide corresponding to fully processed ROAD-1 was synthesized, subjected to folding/oxidation conditions, and purified. ROAD-1 was active against Staphylococcus aureus, Escherichia coli, and Candida albicans in a concentration-dependent manner. We determined the structure of ROAD-1 using NMR spectroscopy and find that the synthetic peptide adopts the canonical disulfide pairing and α-defensin fold. The antimicrobial mechanism of defensins has been correlated with their ability to disrupt and permeabilize the cell envelope, activities that depend on the surface features of the folded peptide. Although ROAD-1 maintains the defensin fold, the oral defensin displays distinct surface features when compared with other α-defensin structures.

Mammalian defensins comprise three structural families (α, β, and θ) of antimicrobial peptides defined by sequence homology and unique disulfide connectivities (1). Representative members of all three families possess activities against a range of microorganisms: Gram-positive and Gram-negative bacteria, and some viruses. In humans, α-defensins are expressed predominantly in leukocytes and in Paneth cells of the small intestine. In addition to their antimicrobial activity in innate immunity, emerging evidence suggests that α- and β-defensins participate in the adaptive immune system, as several peptides have been shown to be chemoattractive for or mobilize immune cells via receptor-mediated signaling pathways (2). An interesting additional role for β-defensins in determining hair color was reported recently expanding the functionality of defensins beyond the immune response (3).

The antimicrobial function of α-defensins is not dependent on the tri-disulfide array, overall topology, cytotoxicity, or cytolytic activities of the peptide (4). Previous studies suggest that defensins interact with the membranes of target pathogen leading to its disruption (5, 6). The initial interaction of the defensins with the microbial membrane is mediated by nonspecific interactions based on surface charges and amphipathicity. Most of mammalian α-defensins have a positively charged surface that is in proximity to, but distinct from, hydrophobic regions of the protein. How these peptides are able to target the membranes of the pathogens and not damage the host cells is an intriguing conundrum. Because the bacterial cell membrane has more anionic lipids, the initial attraction can be electrostatically driven. The most prevalent hypothesis regarding disruption of the microbe involves membrane depolarization following fusion of defensin with membrane and subsequent lysis of the cell (7). However, details of the molecular mechanism are yet to be unveiled.

All defensins are expressed as prepropeptides. The mature peptide results from sequential removal of the signal and pro-segments giving rise to a mature, tridisulfide-containing peptide containing 29–35 (α-defensins), 38–45 (β-defensins), or 18 (θ-defensins) amino acids. Defensin structures have been solved using both x-ray crystallography and NMR. To date, structures for seven α-defensins have been reported: for human neutrophil defensins HNP-1 (8), HNP-3 (9), and HNP-4 (10); rabbit kidney, RK-1 (11); mouse Paneth cell Crp-4 (12); and human Paneth cell HD-5 and -6 (10). These structures display the canonical α-defensin disulfide arrangement and a similar fold, but differ markedly in surface charge distribution and loop sizes/orientations.

Rhesus macaque leukocytes contain eight α-defensin sequences that were previously grouped into two families based on homology to either human neutrophil (HNP-1) or enteric (HD-5) defensins (13). The diversity of the eight peptides is a consequence of two mechanisms; some of the isoforms differ at amino acid positions internal to the mature peptide sequence, whereas others are the result of differential post-translational processing at the α-defensin amino termini. The role of antimicrobial peptides in oral host defense has been an area of active investigation, stimulated in great part by...
characterization of histatins, histidine-rich antimicrobial peptides expressed in human saliva (14, 15). Here we report the first example of an α-defensin expressed in oral tissues of rhesus monkeys and describe its synthesis, structure, and antimicrobial activities.

**EXPERIMENTAL PROCEDURES**

**cDNA Cloning**—Standard methods were used to isolate total RNA from snap frozen necropsy specimens of oral tissues and bone marrow from otherwise healthy adult rhesus macaques (California National Primate Research Center). Three μg of RNA was reverse transcribed in a 20-μl reaction volume using Super Reverse Transcriptase II (Invitrogen). A GenBankTM BLAST search disclosed the presence of 33 *Macaca mulatta* α-defensin gene sequences. To identify the α-defensins expressed in macaque oral tissues, the gene sequences were used to design 12 antisense primers. These were paired with a primer (sense 1, 5'-ATCCCTGTGCTGCCCCCTTCTC-3') corresponding to a conserved region of the signal sequence (within exon 2) of myeloid and enteric defensin genes. One-μl samples of cDNAs derived from rhesus adult bone marrow and that pooled from several oral tissues were amplified, analyzed by agarose gel electrophoresis, gel purified, subcloned, and sequenced. Clones encoding α-defensins were further characterized by 3'-rapid amplification of cDNA ends using a second primer (sense 2, 5'-TCTTCCAGGTGACCCCAGGC-3') corresponding to the 5'-untranslated sequence just upstream of the initiation methionine, and sequenced. Reverse primers 1 and 2 (antisense 1, 5'-GCCCTTGCTCAGGCACAG-3'; antisense 2, 5'-GCTCGCGACGCAAGCAGACT-3') were used with the sense 2 primer to amplify the entire coding sequence corresponding to ROAD-1 and a homologous bone marrow α-defensin, respectively. Assembled sequences obtained after subcloning provided the complete coding sequence of each α-defensin reported here.

**Nucleotide Sequence Accession Numbers**—The cDNA sequences for the following rhesus α-defensins have been submitted to GenBank and assigned the numbers indicated: ROAD-1, EU856519; ROAD-2a, EU856520; ROAD-2b, EU856521; and rhesus myeloid α-defensin (RMAD)-9, EU856522.

**Peptide Synthesis**—The ROAD-1 mature peptide chain was assembled on polyethylene glycol resin using a continuous flow 9050 Plus PepSynthesizer (Millipore) essentially as described in Ref. 16. The synthesis utilized N-(9-fluorenyl)methoxycarbonyl (Fmoc) chemistry, Trt/tBu/Pbf side chain protection, and O-pentafluorophenyl preactivated form. The peptide was deprotected and cleaved from the resin with Reagent-K for 4 h, and this product was extracted with 50% acetic acid, water and the aqueous phase was lyophilized. The crude material was reduced with dithiothreitol and purified on a 45 × 300-mm Vydac C18 cartridge column developed at 60 ml/min with linear water, acetoniitrile gradients containing 0.1% (v/v) trifluoroacetic acid. Peak fractions were analyzed by MALDI-TOF MS, analytical HPLC, and acid-urea PAGE. Purified samples were pooled, vacuum concentrated, and stored at 4 °C prior to oxidation and folding.

**Forging of Reduced ROAD-1**—A 0.1 mg/ml solution of reduced ROAD-1, dissolved in 0.1% (v/v) acetic acid, was alkalinized to pH 7.8 by dropwise addition of ammonium hydroxide. Air oxidation was carried out by stirring the solution at room temperature overnight in an open flask. This solution was acidified to pH 2, and the oxidized peptide was purified on a preparative C18 HPLC column. Fractions were analyzed by MALDI-TOF mass spectroscopy and in the main peak was found to have the mass of the theoretically oxidized peptide (3740.5 Da). This material was lyophilized giving a white fluffy powder.

**Antimicrobial Assays**—Escherichia coli ML35, *Staphylococcus aureus* 502a, and *Candida albicans* 16820 were utilized as target organisms in agar diffusion assays as previously described (17). Briefly, organisms were grown to mid-log phase in trypticase soy broth (bacteria) or Sabouraud dextrose broth (C. albicans). Organisms were seeded at 1 × 10⁶ in 1% agarose agar buffered with 10 mM PIPES, pH 7.4, containing 0.03% trypticase soy broth or Sabaraud agar and poured into a 9-cm² square Petri dish. Five-μl aliquots of each peptide, dissolved in 0.01% acetic acid at 10–300 μg/ml, were added to 10-μl wells. After incubation at 37 °C for 3 h, the seeded agar was overlaid with warmed agarose containing 6% nutrient broth. Plates were incubated at 37 °C for 18–24 h. HNP-1 was purified from acid extracts of human neutrophils using methods described in Tang *et al.* (13) for the purification of RMAD-1. The identity and purity of HNP-1 were determined by MALDI-TOF MS. Antimicrobial activity was determined by measuring the zones of clearing around each well and plotting the diameter as a function of peptide concentration.

**NMR Spectroscopy**—NMR samples were prepared by dissolving 1 mg of synthetic ROAD-1 in 0.35 ml of H₂O and adjusting the pH to between 3.5 and 4.0. Experiments were performed on a Varian Inova 800 MHz NMR spectrometer. Data were collected on the sample in 90% H₂O, 10% D₂O. Following these experiments, data were collected again after the sample was lyophilized and resuspended in an equal volume of 100% D₂O. Data were immediately collected to determine slowly exchanging backbone signals. NOESY (τm 250 and 75 ms), DQF-COSY (τm 75 ms), and DQF-COSY experiments were performed; the water peak was eliminated using WATERGATE. Data (1024 × 256 complex points) were processed using NMRPipe (18) and spectra were analyzed using the software Analysis (19).

**Structure Calculations**—Structure calculations were carried out using the *ab initio* simulated annealing protocol of the Xplor-NIH program, version 2.17 (20, 21). Structures were calculated on the basis of distance restraints derived from the NOE cross-peaks and dihedral angle constraints derived from the

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3 The abbreviations used are: ROAD, rhesus macaque oral α-defensins; Crp, creatinid; DQF-COSY, double quantum filtered-correlation spectroscopy; HD, human defensin; HNP, human neutrophil peptide; HPLC, high performance liquid chromatography; MS, mass spectroscopy; MALDI-TOF, matrix-assisted laser desorption/ionization time of flight; NOESY, nuclear Overhauser effect spectroscopy; Pbf, 2,2,4,6,7-pentamethyldihydrobenzo[furan-5-sulfonyl; PIPES, piperazine-1,4-bis(2-ethanesulfonic acid); RK, rabbit kidney; RMAD, rhesus macaque myeloid α-defensins; tBu, t-Butyl; TOCSY, total correlated spectroscopy; Trt, trityl; r.m.s., root mean square; HA, alpha proton.
coupling constant data. Hydrogen bond constraints were incorporated during the refinement step. NOE intensities were qualitatively categorized to strong, medium, and weak and translated to the upper bound distance limits to 2.5, 3.0, and 4.0, respectively. Sum averaging method was used for the NOE potential energy calculations.

The initial coordinates of ROAD-1 were generated with random $\phi$ and $\psi$ angles and the extended side chain using the parameters in the protein-1.0.par file of the Xplor-NIH program. To begin the calculations the observed disulfide bonds between the cysteine residues were incorporated as disulfide bridges and were later changed to disulfide bonds during refinement. The starting structure was energy minimized (Powell minimization; 1000 steps) to ensure that the structure met covalent requirements.

Simulated annealing was performed in three steps, a 20-ps high temperature dynamics at 3500 K with experimental restraints but with no Van der Waals term followed by a 100-step cycle or 0.2 ps with Van der Waals turned on and then a gradual cooling to 25 K with a time step of 12.5 ps. During the 100-step cycle the Van der Waals radius and force constants were incrementally raised. Also the force constants for angles and improper dihedral angles were changed in this cycle. In the cooling step, all the weights were incrementally raised to their final values. Electrostatic terms involving the side chain-side chain interactions were excluded from the energy function during refinement so as not to place undue emphasis on charge-charge interactions in the absence of solvent molecules in the calculations (21). The structure generated was subjected to 500 steps of energy minimization using the conjugate gradient Powell algorithm.

Several rounds of calculations were performed with each round generating 50 structures. After each cycle the structure with least violations was used as the beginning structure for subsequent calculations. Protein structures were analyzed using PROCHECK (22, 23) and displayed using Chimera (24). MOLMOL (25) was used for displaying the electrostatic surface of ROAD-1. The coordinates representing the solution structure of ROAD-1 and the experimental restraints have been submitted to the Protein Data Bank with access code 2K1I.

RESULTS

**Discovery of ROAD-1 and Related $\alpha$-Defensins**—To determine which of the known rhesus macaque $\alpha$-defensin genes are expressed in the oral cavity, we characterized reverse transcriptase-PCR products using mRNA from cheek pouch, esophagus, gingiva, lip, parotid gland, submandibular gland, tongue, and tonsillar necropsy tissues from adult monkeys. Initially, cDNAs from each source were pooled and screened for $\alpha$-defensin sequences in PCR using 12 primer pairs (see “Experimental Procedures”). Oral cDNAs were amplified, subcloned, and 23 clones were sequenced disclosing five $\alpha$-defensin coding sequences. Four of these corresponded to known myeloid $\alpha$-defensins: RMADs 1, 2, 3, and 4 (13). The fifth $\alpha$-defensin sequence corresponded to a new peptide, here termed ROAD-1, which was not among those previously detected in macaque leukocytes (13) or small intestine (26). The full-length coding sequence of the ROAD-1 cDNA, determined by 3’-rapid amplification of cDNA ends and PCR (see “Experimental Procedures”), encodes a 93-amino acid $\alpha$-defensin prepeptide that is most similar to the precursor encoding RMAD-4; the two peptides are 92.5% (87/94) identical at the amino acid level (Fig. 1). Of note is the absence of one amino acid between the second and third cysteines in the ROAD-1 primary structure, as compared with most known primate myeloid and enteric $\alpha$-defensins (e.g. see RMAD-4 in Fig. 1).

Using ROAD-1 sense 2 and antisense 1 (see “Experimental Procedures”), we amplified a number of related cDNAs derived from macaque cheek pouch mRNA. The sequences of the amplicons disclosed the expression of ROAD-1 and two additional oral $\alpha$-defensins, termed ROAD-2a and -2b. As shown in Fig. 1, ROAD-2a and -2b have identical mature peptide sequences, and differ from mature ROAD-1 by a single amino acid (Phe $\rightarrow$ Ser) six residues from the carboxyl terminus. Interestingly, the signal and pro-segment sequences of ROAD-2a and -2b differ at six residue positions (Fig. 1).

In parallel with the PCR-based screen for oral $\alpha$-defensins, we characterized bone marrow cDNAs using the 12 primer pairs, both as a positive control for known myeloid $\alpha$-defensins, and to determine which oral $\alpha$-defensins were also expressed in myeloid elements. In bone marrow cDNA we detected all of the previously characterized rhesus myeloid $\alpha$-defensins (RMAD 1–8) and also discovered a new $\alpha$-defensin, termed RMAD-9 that, like the ROADS is most similar to RMAD-4 (Fig. 1). However, based on the absence of the RMAD-9 cDNA sequence in pooled oral cDNAs, it appears that RMAD-9 is not expressed in the oral cavity.

We produced a synthetic version of mature ROAD-1, assembled as a 32-residue chain that was purified as the fully reduced peptide, then folded and oxidized in room air, and repurified by HPLC. The final product had the correct mass (3740.3 Da [M + H]+; calculated = 3740.3 Da), and eluted on analytical reverse phase-HPLC as a single peak but eluted much earlier than RMAD-4 (Fig. 2). ROAD-1 also migrated more rapidly than RMAD-4 in acid-urea PAGE, which was unexpected given that ROAD-1 (32 residues) and RMAD-4 (33 residues) are very similar in size and have the same net charge at values below pH 6. **Antimicrobial Activities of ROAD-1**—Synthetic ROAD-1 was evaluated for its antibacterial and antifungal activities in an agar
diffusion format, and in these assays was compared with RMAD-4 and human α-defensin HNP-1. As shown in Fig. 3, all three peptides showed a dose-dependent antimicrobial effect against *S. aureus*, *E. coli*, and *C. albicans*. However, ROAD-1 produced the greatest zone of clearing against all three targets. Of note was the relative antimicrobial potency of the peptides against *E. coli*: ROAD-1 was approximately five times more active than RMAD-4 or HNP-1 against this Gram-negative organism (Fig. 3). Using suspension assays (13), we demonstrated that each peptide killed the same panel of test organisms, indicating that the clearing observed in the diffusion assays (Fig. 3) was at least partly due to a microbicidal effect (data not shown).

**NMR Spectroscopy and Analysis**—Two-dimensional homonuclear NMR experiments were recorded on the peptide at 800 MHz. The two-dimensional NMR spectra of ROAD-1 acquired at 30 °C in 90% H2O, 10% D2O demonstrated very good chemical shift dispersion indicating a well structured protein. NMR diffusion measurements predict ROAD-1 to exist as a monomer in solution (data not shown). Using standard homonuclear methods (27), a near complete proton assignment was obtained. The DQF-COSY spectrum was used to identify spin systems and TOCSY and NOESY spectra were used to determine sequential connectivity along the backbone (Fig. 4). The side chain H2 of Arg2 was observed to be shifted downfield at 9.64 ppm compared with the expected value of 7.30 ppm. This is very similar to the corresponding Arg in the published data on mouse cryptdin-4 (28). Spectra obtained at a slightly different temperature, 25 °C, were used to resolve overlapping peaks and to confirm all resonance assignments. DQF-COSY and NOESY spectra acquired on a sample transferred to 100% D2O were used to resolve signals under the water resonance and to identify the residues involved in hydrogen bonding. The ROAD-1 sequence contains six cysteines at positions: 4 (CysIV), 6 (CysIII), 10 (CysIII), 20 (CysIV), 30 (CysIV), and 31 (CysV) and their positions are very similar to those reported for other α-defensins. NOESY spectra showed several inter-cysteine NOEs that establish disulfide bond pairs such as 4:CysHA to 31:CysHA; 6:CysHB1/2 to 20:CysHB1/2; 10:CysHA to 30:CysHB1/2 implicating the disulfide bonds in ROAD-1 to...
follow the canonical α-defensin bond pattern, i.e. Cys¹–Cys⁶, Cys²–Cys⁷, and Cys³–Cys⁸.

The deviation of HA chemical shift from the random coil shifts can be readily used to identify the secondary structure elements in a protein (29). Fig. 5 shows the chemical shift index and also gives a summary of the inter residue NOE connectivities. The predominance of positive values in the chemical shift index is consistent with the β-sheet secondary structure we find in ROAD-1, typical of an α-defensin fold (2). The HA to HN_i+1 NOEs analyzed for the resonance assignments gave a strong indication that residues 3–7, 14–22, and 26–31 form β sheets. Numerous long-range NOEs between residues (such as those between residues, e.g. 7 and 28, and 19 and 26) support the arrangement of the three β sheets in an anti-parallel conformation.

**Solution Structure of ROAD-1**—A total of 473 distance restraints derived from NOESY data and 21 dihedral restraints were used for the structure calculations. Nine hydrogen bonding restraints were added during the refining step. All the structures were calculated using simulated annealing and refined without explicit solvent. Of a total of 50 structures, 10 structures were selected to represent the solution structure of ROAD-1 based on lowest energy and least restraint violations; these are shown in Fig. 5B. The statistical data summarizing the quality are presented in Table 1 and reveal good convergence. All the selected structures have NOE violations less than 0.5 Å and dihedral violations less than 5 degrees. In addition, the structures show correct covalent geometries as evident from low deviations from the standard bond lengths and angles and in consideration of Ramachandran statistics. Moreover, the superposition of backbone atoms of all 10 low
energy structures shows very low deviation. The root mean square (r.m.s.) deviations for backbone atoms (N, C, and C') for residues 3–29 is 0.86 Å. The backbone r.m.s. deviations of 1.35 Å for residues 1–32 suggests that the terminal residues are flexible with the NH₂ terminus appearing to be more disordered than the COOH terminus (Fig. 5B).

The predominant secondary structure in ROAD-1 is β sheet. The three strands are composed of residues: 3 to 7, 15 to 21, and 26 to 31, arranged in an anti-parallel manner (Fig. 6B). The NH₂-terminal β strand (referred to as β1 from hereon) has a twist at His⁵, most likely as a consequence of being positioned between two cysteine residues (CysI and CysII) with backbone conformations restricted by disulfide bonds to remote cysteines (CysVI and CysIV). β1 is connected to the second β strand (β2) by two turns: a type IV turn (residues 8 to 10) followed by a type I turn (residues 11 to 14). The disulfide bond between CysIII and CysV stabilizes the type I turn. β2 is followed by a tight hairpin turn (residues 22 to 25) connecting it to the COOH-terminal β strand (β3).

The solvent accessibility of each residue in the peptide was probed by rolling a sphere of radius 1.4 Å over the protein surface (Fig. 7). Although there are variations in sequence length and turn conformations, the total solvent accessible surface areas are very close (within 6%) for HNP-3, HD-5, Crp-4, RK-1, and ROAD-1. ROAD-1 and HD-5 have slightly larger solvent accessible surface areas as compared with Crp4, HNP3, and RK-1. A comparison of solvent accessible surface areas per residue for ROAD-1, HD-5, Crp-4, and RK-1 reveal a similar pattern. The total solvent accessible surface area of RMAD-4 is comparable with ROAD-1; however, NMR hydrogen exchange results of RMAD-4⁴ suggests that RMAD-4 is more flexible than ROAD-1.

⁴ S. Vasudevan and M. J. Cocco, manuscript in preparation.
DISCUSSION

We report the identification of four new rhesus macaque α-defensins, three of which (ROADs) are expressed in oral mucosa, and RMAD-9, a new myeloid α-defensins. ROAD-1, selected for further characterization, was synthesized and its structure and activity were determined. ROAD-1 adopts an α-defensin fold and was found to be both antibacterial and antifungal in agar diffusion assays. As noted, ROAD-1 differs from RMAD-4 in only four positions; these are depicted on the structure in Fig. 6B. These amino acid substitutions do not change the net charge under acidic conditions. However, we see a difference in migration of ROAD-1 compared with RMAD-4 in acid urea PAGE (Fig. 2). Notably, we also find that ROAD-1 is more potent than RMAD-4 in killing of S. aureus and C. albicans at similar concentrations (Fig. 3). Thus the change in the four residues results in an observable change in activity and surface properties.

It was previously shown that the myeloid α-defensins from rhesus macaque contain two subfamilies that are highly similar to either human myeloid (HNP-1 to -3) or enteric (HD-5) defensins at the protein and DNA level. A pairwise BLASTP (31) comparison of the ROAD-1 sequence shows that it has 60% sequence identity to human enteric α-defensin 5 (HD-5). The sequence of ROAD-1 including its propeptide shows very high (87%) sequence identity to RMAD-4. The homology to HD-5 and RMAD-4 places ROAD-1 in the second family, along with RMAD-5, -6, and -7 (13). The four-residue variant between ROAD-1 and RMAD-4 are all located within a radius of 7 Å on one face of the ROAD-1 structure (Fig. 6B).

ROAD-1 adopts a typical α-defensin fold with the core structure made of three-stranded β sheets stabilized by three intramolecular disulfide bonds (2). The β sheet in the ROAD-1 structure appears to be twisted like all other α-defensin structures (9); residues Gly^{18} and Phe^{27} enhance this twist by forming a β bulge. Fig. 8 shows a superposition of solution structure of ROAD-1 with that of four other α-defensins, Crp-4 (12), HNP-3 (9), HD-5 (10), and RK-1 (11). The length, shape, and the orientation of the hairpin turns are different in all the five structures. The conformations of segments connecting β1 and β2 or β2 and β3 are a prominent difference among defensins. Similar to ROAD-1, RK-1 β1 is connected to β2 via type IV and I turn,
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whereas in Crp-4 the β1 is connected to β2 by γ turn and a type II turn. The β-hairpin loop between β2 and β3 is conserved throughout all the mammalian, insect, and plant defensins (32) and could influence the function of defensins because there is evidence that this β-hairpin loop is the active region of the rabbit neutrophil defensins NP-2 molecule (32). The hairpin turn is responsible for changing the presentation of side chains of residues with significant consequences depending on its intervening length. For example, in ROAD-1 the turn positions the side chain of Arg25 on the same side as Phe25 (similar to HD-5 where Arg25 is on the same side as Tyr27), whereas in Crp-4, the shortened turn positions the Arg24 in the opposite side of Phe25 and Tyr27. The variations in backbone positions between the five structures overlaid in Fig. 8 are significant and larger than the errors for individual structures. Threading a novel defensin sequence through any of these structures is likely to produce a model that is close but not identical to the correct structure. Moreover, structural data are necessary to correctly define side chain rotamer conformations.

Regardless of activity in membrane disruption or potential signaling, defensin function is a property of its surface. We find similar accessible surface areas among different sequences (Fig. 7). Generally, most of the defensin residues are accessible and consequently capable of recognition events with other proteins or lipid. Notably, Glu13/Glu14/Glu15 has the least accessible surface area of all α-defensins discovered so far report the presence of a salt bridge between Arg and Glu residues aligned with positions 7 and 14 in ROAD-1. Arg7 and Glu14 are located at the two ends of the loop connecting β1 and β2. Weak NOE contacts between the side chains of Arg7 and Glu14 show they are in proximity making a salt bridge connection between either or both of oxygen atoms of Glu14 and the H8 atom of Arg7 possible (Fig. 6A). This salt bridge could stabilize loop formation between the disulfide bridge of Cys10 and Cys20. Upon analysis it was found that five of the 10 conformers in the ensemble have the Glu oxygen and the Arg H8 atom within a distance of 4 Å. A similar observation was made for the NMR structure of Cyanovirin-N where the salt bridge between Glu68 and Lys38 exits in only five conformers of 40 in the NMR conformer ensemble (34). Two pieces of evidence strongly support the presence of a salt bridge in ROAD-1: first, the side chain Arg H8 protons are detected, these usually exchange too fast to be detected by NMR spectroscopy, second, the unusual chemical shift for the H8 atom in Arg7 (9.5 ppm, Fig. 4) suggests a different chemical environment for this residue as compared with the typical exposed arginine and is consistent with involvement in a salt bridge. The conservation of Glu14 across most of the α-defensins (only 3 exceptions are observed) implies a structural, functional, or evolutionary role for this residue. In the case of HNP-3 the salt bridge is known to impart resistance to proteolysis by elastase (35).

The electrostatic surface (Fig. 6C) demonstrates the amphipathicity of this protein. ROAD-1 contains eight Arg distributed fairly evenly in the primary sequence; however, when the protein is folded most of these basic residues are located on one face. The positively charged surface is distinct and separate from the hydrophobic region. A small negatively charged patch from the Glu14 side chain and the nearby COOH terminus appear at the interface of positively charged surface and the hydrophobic region. The ROAD-1 electrostatic surface is similar to that reported for RK-1 (Fig. 8 in Ref. 11). However, other defensin structures show very different patterns of charge distributions. The variations in α-defensin electrostatic surface distributions suggest that even though they have an overall similar topology they could have distinct mechanisms for their mode of antimicrobial action. For example, HNP-4 was found to be active against E. coli, Streptococcus faecalis, and C. albicans, whereas HNP-5 did not show any activity against S. faecalis (33).

Several antimicrobial peptides that kill bacteria and fungi are known to function by lysis of microbial membranes. The amphipathic nature of α-defensins makes it possible for them to permeabilize and disrupt membranes. The basic residues in defensins can interact electrostatically with the negatively charged membrane phosphate groups, leading to defensin anchoring into the membrane bilayer. We can hypothesize that the large hydrophobic surface in ROAD-1, consisting of the side chains of Ile22, Gly24, Ile36, and Phe27, is a good candidate for hydrophobic interactions with the membrane hydrocarbon. It is worth noting that α-defensins from different tissues in rhesus macaque have subtle changes in protein sequence. This variation in protein sequence with minimal changes.

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