Structural Requirements for Potent Versus Selective Cytotoxicity for Antimicrobial Dermaseptin S4 Derivatives

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To better understand the structural requirements for selective cytotoxicity of antimicrobial peptides, seven dermaseptin S4 analogs were produced and investigated with respect to molecular organization in solution, binding properties to model phospholipid membranes, and cytotoxic properties. Native dermaseptin S4 displayed high aggregation in solution and high binding affinity. These properties correlated with high cytotoxicity. Yet, potency was progressively limited when facing cells whose plasma membrane was surrounded by increasingly complex barriers. Increasing the positive charge of the native peptide led to partial depolymerization that correlated with higher binding affinity and with virtually non-discriminative high cytotoxicity against all cell types. The C-terminal hydrophobic domain was found responsible for binding to membranes but not for their disruption. Truncations of the C terminus combined with increased positive charge of the N-terminal domain resulted in short peptides having similar binding affinity as the parent compound but displaying selective activity against microbes with reduced toxicity toward human red blood cells. Nuclear magnetic resonance-derived three-dimensional structures of three active derivatives enabled the delineation of a common amphipathic structure with a clear separation of two lobes of positive and negative electrostatic potential surfaces. Whereas the spatial positive electrostatic potential extended considerably beyond the peptide dimensions and was required for potency, selectivity was affected primarily by hydrophobicity. The usefulness of this approach for the design of potent and/or selective cytolytic peptides is discussed herein.

After a half-century of virtually complete control over microbial infections, the 1990s have brought a worldwide resurgence of infectious diseases by evolution of antibiotic-resistant strains. So far, resistance has developed to nearly all antimicrobial drugs (1, 2). Levels of resistance to antibacterial agents, for instance, are increasing at an alarming rate as antibiotics or possibly even be used as an alternative (6, 7). So far, more than 400 ribosomally made antimicrobial peptides have recently emerged as a potential class of novel antimicrobial agents that could either complement existing antibiotics or possibly even be used as an alternative (6, 7).

Antimicrobial peptides are often described as membrane-active agents although their detailed mechanism of action is not well understood. Nevertheless, several lines of evidence exist that enable us to stipulate a probable mechanism. Thus, isomers composed of D-amino acids only display identical cytotoxic potency as the L-enantiomers, implying that their mechanism of action is not mediated by interaction with a stereospecific target such as protein receptors, enzymes, and so on (20, 21). Rather, it seems that the peptide physicochemical properties (i.e. charge and hydrophobicity) are the main factors affecting antimicrobial activity. Indeed, despite a large heterogeneity in primary and secondary structures, all antimicrobial peptides carry a net positive charge; they can fold into well defined amphipathic structures, and they often display strong lipophilic properties and activity against a wide range of microorganisms (6–11). In addition, microbicidal activity exhibits strong concentration dependence. At lower concentrations, the peptides are virtually inactive until a critical concentration is reached (22, 23) suggesting that the active form of the peptide is an oligomer. Some of these peptides were stipulated to form ion channels or pores, as suggested by their ability to dissipate the electric potential across energy-transducing membranes and ion conductance across lipid bilayers (24–30).

Despite the fact that the surrounding environment of the target cells plasma membrane is extremely diverse, very few mechanistic studies take into account how the mode of action of
the peptides might be affected by problems relating to the ability of the peptides to access the cell membrane. For instance, a microbial membrane enclosed within a cell wall would be less accessible than the virtually “naked” membrane of protozoan cells. Correspondingly, potent antiprotozoan peptides were shown to display weak antibacterial activity (21). Furthermore, many antimicrobial peptides display a broad spectrum of activity (31–33), yet many are quite inactive on normal eukaryotic cells. The basis for this discrimination is also unclear. It appears to be related to the lipid composition of the target membrane (i.e. fluidity, negative charge density, and absence/presence of cholesterol), and the possession, by the peptide-susceptible organism, of a large negative trans-membrane electrical potential (34).

Thus, whereas the precise mechanism of action of antimicrobial peptides is yet to be better defined, the microbicidal effect is widely believed to result from their capacity to permeabilize the membrane of target cells. Such a mechanism of action endows the peptide-based antimicrobial system with attractive advantages over classical antibiotics because it makes it extremely difficult for microbes to develop resistance (18, 35, 36). However, a major downside of such a mechanism is reflected in its unsuitable activity over a wide range of cell types, which could be problematic, for instance, in systemic routes of administration (37). In some ironic way therefore, a major challenge of this field of research consists of figuring out how to endow specificity to a system that by definition is nonspecific.

Toward better understanding the mechanism of action of peptide-antimicrobials, particularly with respect to better defining the factors affecting potency versus selectivity, we investigated in this study the relations between physicochemical properties of the peptides (composition, three-dimensional structure, and molecular organization in solution) and their interaction with target membranes as a function of the accessibility of the membrane. The 28-residue antimicrobial peptide dermaseptin S4 was selected for this investigation. Dermaseptin S4 belongs to a large family of linear polycationic peptides from frogs (12, 13, 38) displaying cytolytic activity in vitro against a large spectrum of pathogens (21, 36, 38–44). Among natural dermaseptins, S4 is unique in displaying high cytotoxicity toward erythrocytes as well (21, 39, 41). Previous structure-activity relationship investigations suggested that peptide aggregation in solution, because of hydrophobic interactions at the peptide extremities, had dramatic consequences on cytotoxic properties including selectivity of the peptides (21). Thus, tampering with the composition of the peptide extremities led to disaggregation and selective activity in vitro and in animal models for infectious disease (36, 44). Yet, the lack of three-dimensional structural data made it impossible to draw sound conclusions as to how the properties of the non-aggregated derivatives contributed to selectivity (21). Here, we investigated the three-dimensional structure of selected dermaseptin S4 derivatives. Because peptide structure might be medium-sensitive, as indicated by circular dichroism measurements (12, 13, 31), the choice of medium for structural studies is critical. We chose to look at the peptide structure a fraction of time prior to the initial interaction (i.e. the electrostatic adhesion) that we believe to represent a most crucial moment in the mechanism of cytolytic action. To mimic such an interface milieu, structural studies were performed in an aqueous medium somewhat more hydrophobic than water, i.e. 20% trifluoroethanol/water (45, 46). We also investigated here the effects of peptide alterations (length, charge, and hydrophobicity) on cytolytic activity as a function of accessibility to the target membrane. The structure-dependent peptide properties were first investigated with respect to binding to model membranes and then correlated with their capacity to lyse erythrocytes and protozoa, for which accessibility is maximal, or fungi, Gram-positive and Gram-negative bacteria, cells for which accessibility is hampered due to the presence of surrounding cell walls of increasing complexity (47).

### EXPERIMENTAL PROCEDURES

#### Peptides

The peptides were synthesized by the solid phase method as described (21) applying the Fmoc (9-fluorenylethoxyxycarbonyl) active ester chemistry on a fully automated, programmable model 433A Peptide Synthesizer (Applied Biosystems). 4-Hydroxyphenylalanine-methoxymethyl-copolymer-1% divinylbenzene resin (Wang resin) and 4-methylbenzyldihydracine resin (Novabiochem) were used to obtain free carboxyl or amidated peptides, respectively. After purification by high pressure liquid chromatography (21) the peptides were subjected to amino acid analysis and mass spectrometry in order to confirm their composition. Peptides were stocked as lyophilized powder at ~20 °C. Prior to testing, fresh solutions were prepared in water, briefly vortexed, sonicated, and centrifuged and then diluted in the appropriate medium. Buffers were prepared using distilled water (Milli-Q, Millipore). All other reagents were of analytical grade.

#### Aggregation in Solution

The aggregation properties of the peptides in aqueous solution were investigated by static light scattering measurements on a PerkinElmer LS-5B Luminescence Spectrometer. Peptides at an initial concentration of 50 μM were successively diluted in 2 ml of PBS (10 mM sodium phosphate, 150 mM NaCl, pH 7.3) at room temperature, and light scattering was evaluated by measuring the reflected light at an angle of 90°, holding both the excitation and the emission at 400 nm (5-nm slits). To describe the dependence of scattered signal on peptide concentration, the intensity of scattered light (mean from at least two independent experiments) was plotted against total peptide concentrations, and a linear regression analysis was performed on the data at the concentration range close to the monomer-micelle transition zone. The static light scattering signal is proportional to the number of aggregated molecules and the size of the aggregate. Therefore, the slope is indicative of the aggregation tendency and reveals the aggregation properties of the peptides, where a slope value above unity indicates the presence of micellar form. For aggregating peptides, the critical micelle concentration (CMC) was evaluated by extrapolating the curve to the intercept with the x axis (48).

#### Binding Experiments

**Liposome Preparation—**Small unilamellar vesicles composed of phosphatidylcholine/phosphatidic acid (PC/PA, 1:1 molar ratio) were suspended in PBS by the extrusion method. Briefly, dry PC and PA were dissolved in EtOH or CHCl3, respectively, and mixed at equimolar ratios. The solvents were evaporated under a stream of nitrogen, and lipids were resuspended in PBS buffer at 0.5 mM total lipid concentration. The resultant suspension was vortexed, briefly sonicated, and passed 21 times through 100 nm polycarbonate membranes in an Avestin LiposoFast-Basic extrusion apparatus to give a translucent solution of vesicles with a mean diameter of 100 nm.

**Surface Plasmon Resonance Analysis—**Peptide binding to phospholipid membranes was determined using the optical biosensor system (BIAcore 3000, Uppsala, Sweden) based on the principle of surface plasmon resonance (49). The sensor chip L1, a carboxymethyl dextran hydrogen derivatized with lipophilic alkyl chain anchors (BIAcore), was used to prepare a lipid bilayer as follows. After washing the chip with octyl glucoside (40 mM), the liposomes (0.5 mM) were injected over the chip surface at a flow rate of 2 μl/min at 25 °C to allow adsorption to the chip. Irregular and loosely bound structures such as multiple lipid layers and partially fused liposomes were washed away by a brief injection of NaOH (50 mM) at a high flow rate (100 μl/min). Bovine serum albumin was used to assess the degree of surface homogeneity. Under these conditions, about 5000 resonance units of lipid were im-

1 The abbreviations used are: PBS, phosphate-buffered saline; PC, egg yolk 1-α phosphatidylcholine; PA, 1-α phosphatidic acid; TFE, trifluoroethanol; TOCSY, total correlation spectroscopy; NOE, nuclear Overhauser enhancement; NOESY, two-dimensional NOE spectroscopy; MOPS, 4-morpholinoethanesulfonic acid; r.m.s.d., root mean square deviation; CMC, critical micelle concentration; MIC, minimal inhibitory concentration.
mobilized which corresponds to a surface density equivalent to 4.6 ng/mm² (50). Surface regeneration between consecutive injections included the sequential injection of NaOH (50 mM) and HCl (50 mM). Association and dissociation rate constants were calculated by nonlinear fitting of the primary data using the BLAevaluation 3.0 software (BLAcore). The association and dissociation rate constants were derived assuming a simple bimolecular interaction model (51). Their ratio (kₐ/kₑ) yields the association affinity constant $K_{a,p}$.

**Cytotoxic Assays**

Hemolytic activity was assessed against human red blood cells. Human blood was rinsed 3 times in PBS by centrifugation for 1 min at 2700 $\times$ g and then resuspended in PBS at 5% hematocrit. A 50-μl suspension containing 2.5 $\times$ 10⁶ red blood cells was added to Eppendorf test tubes containing 200 μl of peptide solutions (serial 2-fold dilutions in PBS), PBS alone (for base-line values), or distilled water (for 100% hemolysis). After incubation (3 h under agitation, 37 °C) samples were centrifuged, and the hemolytic activity was assessed as a function of hemoglobin leakage by measuring the absorbance of 200 μl of supernatant (405 nm). Statistical data were obtained from at least two independent experiments performed in duplicate.

Antiprotozoan activity was assessed against the promastigote form of a Leishmania major clinical isolate (graciously provided by Prof. Kobi Golenzer, the Parasitology Laboratory at Hadassah-Hebrew University, Jerusalem, Israel) cultured in RPMI 1640 complemented with 20% fetal calf serum, 1% penicillin, and 1% streptomycin. Inhibition of proliferation was assessed for a 100-μl suspension of the cells added to a 10-μl volume of test tubes containing 100 μl of culture medium (non-complemented RPMI) in 96-well plates (Nunc) containing zero or various peptide concentrations (serial 2-fold dilutions). After the incubation period (3 h, 27 °C) the number of viable (motile) cells was determined by counting an aliquot from each culture on a Neubauer cell counter under a microscope (Olympus IX70). Statistical data were obtained from at least two independent experiments performed in duplicate.

Antifungal activity was assessed against a clinical isolate of Cryptococcus neoformans (strain B3501, graciously provided by Prof. Itzhac Polacheck, the Mycology Laboratory at Hadassah-Hebrew University, Jerusalem, Israel) cultured in RPMI supplemented with 150 mM MOPS, Inocula of 10⁶ cells/ml were used. The cell populations were estimated by absorbance measurements at 620 nm after incubation overnight at 30 °C. Statistical data were obtained from at least two independent experiments performed in duplicate.

Antibacterial activity was assessed against clinical isolates (graciously provided by Dr. Yehuda Carmeli, Division of Infectious Diseases, Sourasky Medical Center, Tel Aviv, Israel) of Staphylococcus aureus (strain B38302) and Escherichia coli (strain U16318) representatives for Gram-positive and Gram-negative bacteria, respectively. Growth inhibition was assessed essentially as described for the antifungal assay except that the culture medium used was 2% tryptone, 10 g/liter yeast extract, 5 g/liter NaCl, pH 7.4, and incubations were performed at 37 °C.

**CD Measurements**

CD spectra in millidegrees were measured with an Aviv model 62A DS spectrometer (Aviv Associates, Lakewood, NJ) using a 0.020-cm rectangular Q5 Helma cuvette at 25 °C (controlled by thermoelectric Peltier elements with an accuracy of 0.1 °C). The CD spectrum was scanned for peptide samples (290 μM determined by UV using standard curves of known concentrations for each peptide) that were dissolved in 20% (v/v) trifluoroethanol/water. CD data represent average values from three separate recordings.

**NMR Measurements**

For the NMR studies, a 1 mM solution of each S4 derivative (Table I) was prepared by dissolving lyophilized powder in an aqueous solution containing 20% (v/v) trifluoroethanol-d₂ (Aldrich) at the apparent pH = 2.4-2.5. NMR experiments were carried out on a Bruker Avance 600 MHz DMX spectrometer operating at the proton frequency of 600.13 MHz using a 5-mm selective probe equipped with a self-shielded xyz gradient coil. The transmitter frequency was set on the HDO signal. The residual water resonance was suppressed using a Watergate sequence (52) for TOCSY experiments and by low power cw irradiation during the relaxation delay for the double quantum filtered COSY and the NOEYSY experiments, and the mixing time of the latter. Two-dimensional homonuclear spectra were acquired in the phase-sensitive mode with 2K complex data points in $t_{1}$ and 400 or 512 $t_{2}$ increments depending on the experiment. The spectral width was ±13 ppm, and the delay relaxations were 0.4 and 2 s in the TOCSY and NOEY experiments, respectively. TOCSY spectra were recorded using the MLEV-17 pulse scheme for the spin lock at several isotropic mixing periods ranging over 50–110 ms with 48–88 scans per $t_{1}$ increment depending on the peptide used (53). The NOEYSY experiments were collected with mixing times varying from 100 to 550 ms in order to gain maximal NOE buildup with partial averaging of the distribution from spin diffusion. The build-up curves were monitored for several well resolved backbone amide protons and the tryptophan aromatic protons when present (Table I). Between 112 and 244 transients were acquired for each $t_{1}$ value for structural analysis. The water signal was used as a reference for all samples, and its chemical shift was corrected at low temperatures relative to external trimethylsilyl propionate (sodium salt; Cambridge Isotope Laboratories) as the zero reference.

The NH-CoH scalar couplings were obtained by fitting the Lorentzian line shapes (Aurelia program) to the in-phase doublets in TOCSY spectra collected with 8K points in the $F_{2}$ dimension and zero-filled to 16K points. For several overlapping signals it was impossible to derive $^{3}$NH₂ couplings with an adequate fit (Fig. 4). Spectra were processed and analyzed with the XWINNMR and Aurelia software packages (Bruker Analytische Messtechnik GmbH) on Silicon Graphics Indy R4000 and Indigo2 R10000 workstations. Zero filling in the $t_{1}$ dimension and data apodization with a shifted squared sine bell window function in both dimensions was applied prior to Fourier transformation. The base line was further corrected in the $F_{2}$ dimension with a quadratic polynomial function.

**NOE Measurements and Experimental Distance Restraints**

NOEYSY spectra used for the determining structural organization of the S4 dermaseptin short and substituted derivatives (Table I) in 20% TFE/water solution were recorded at 285 K. A more elevated temperature of 310 K was required to obtain an adequate spectrum for the $K_{d}$ K₆-S4 analog because at lower temperatures severe overlap of the resonance lines was observed, presumably due to the aggregation of the peptide. The mixing time was set to 250 ms for all peptides, although the mixing times for the last two peptides were decreased to 6 ms in order to avoid contribution of spin diffusion for all the peptides used in this study (see above). Furthermore, NOEYSY spectra of the short derivatives collected at longer mixing times did not reveal any additional significant NOE connectivities. Integrated peak volumes were converted into distance restraints using an $\alpha$-4 dependence and were calibrated relative to the fixed distance of 2.68 Å between the two adjacent protons of the tryptophan aromatic ring. Pseudo multiple-proton NOE constraints were derived from the methyl groups (54, 55). For the structural calculations, a total of 181 distance restraints were used for $K_{d}$ K₆-S4 peptide, among which 66 were sequential and 47 were between $i$ and $i+2$, $i$ and $i+3$ and $i$ and $i$ pairs of interacting protons (for details see Fig. 4). Among the 131 restraints obtained for the $K_{d}$ S₄-S₄(1-13)a, gave a total 146 restraints comprising 46 intra-residual, 59 sequential, and 41 medium range restraints stemming from the NOE connectivities between residues further than two residues apart. The shortest analog, K₆-S₄(1-13)a, gave a total 146 restraints comprising 46 intra-residual, 59 sequential, and 41 medium range restraints.

**Structure Calculation**

The structures were calculated by the hybrid distance geometry-dynamical simulated annealing method using XPLOR version 3.856 (56). The NOE energy was introduced as a square well potential with a force constant of 50 kcal/molÅ² that was kept constant throughout the protocol. Each round of simulated annealing refinement consisted of 1500 3-fs steps at 1000 K and 3000 1-fs steps during cooling to 300 K. Finally the structures were minimized using conjugate gradient energy minimization for 4000 iterations. These initial low resolution structures were used to assign stereospecifically ambiguous peaks. The InsightII program (Molecular Modeling System version 97.0, Molecular Simulations, Inc.) was used for visualization and analysis of the NMR-derived structures. Their quality was assessed using Procheck (57).

Electrostatic free energies were derived from finite difference solutions of the Poisson-Boltzman equation using the DelPhi program from within the InsightII software package (58). The AMBER forcefield (59) was employed using Van der Waals radii, and a full Coulombic calculation was performed using a boundary extending 10 Å beyond the longest axis of the peptide. The internal peptide dielectric constant was
5.0; the water dielectric constant was 80, and its radius was 1.4 Å. When a dielectric constant of 60 was used to reflect the addition of trifluoroethanol, no significant change in the calculated electrostatic potential distribution resulted. An energy convergence criterion of $1 \times 10^{-6}$ kcal/mol was applied.

RESULTS

Toward a better understanding of the structural requirements for selective cytotoxicity, we generated a series of dermaseptin S4 derivatives (the peptide sequences are listed in Table I). Peptide purity was in the range of 95 to $>99\%$, as determined by analytical high pressure liquid chromatography, and their identity was confirmed by mass spectrometry and amino acid analysis (data not shown). The peptides were investigated with respect to organization in solution, binding properties to model phosphomembranes, and cytotoxic properties, and the three-dimensional solution structure of selected peptides was determined.

**Aggregation in Solution**—Peptide aggregation was investigated using their static light scattering properties in PBS as a function of the range of concentrations used for bioassays (up to 50 $\mu$M). Fig. 1 shows the concentration-dependent light scattering profile of the native dermaseptin and its derivatives. The results indicate that dermaseptin S4 is in the highest aggregation state (slope = 12, a slope of 1 is expected for monomers) with a CMC value of 0.2 $\mu$M as estimated from the intersection with the peptide concentration axis (inset of Fig. 1). This is in close agreement with the value found (0.1 $\mu$M) using a fluorescence method (39). The truncated derivatives, S4(9–28) and S4(5–16), did not display aggregative properties. This is indicated by the linear relationship between their light scattering and concentrations (slopes = 0.8–0.9).

K$_4$K$_{20}$-S4 was clearly less aggregated than native dermaseptin S4 (slope = 7.4 with the CMC estimated at 0.3–0.4 $\mu$M) suggesting that the introduction of a positive charge in each of the hydrophobic domains was responsible for limiting intermonomer interactions. Similarly, replacing the 12 C-terminal residues of K$_4$K$_{20}$-S4 with a carboxamide resulted in additional loss of aggregation. Further truncating the C terminus of the peptide by 3 (or 6) residues resulted in a similar outcome; both K$_4$S4(1–13)a and K$_4$S4(1–10)a displayed the light scattering profile expected for monomers.

**Binding Experiments**—To investigate the lipophilic properties of the peptides, their association affinity constant ($K_{app}$) was determined using the BIAcore system whereby small unilamellar vesicles composed of PC/PA were adsorbed onto a sensor chip (L1) to form a supported lipid bilayer, which chemically and physically resembles the surface of a cell membrane. Fig. 2 portrays a typical binding (association/dissociation) profile for various concentrations of dermaseptin S4 from which the $K_{app}$ was derived. Nonlinear analysis of the sensorgram data was performed assuming a simple bimolecular association model (60) as used previously to describe the binding affinity of the antimicrobial peptide cerneol (61). Although this represents a certain simplification, a satisfactory fit of the binding curves to this model was obtained. Because the level of immobilized membrane varied slightly between the injection cycles, the maximal response ($R_{max}$) was set to a local parameter, $i.e.$ unique for each sensorgram. The resulting data are summarized in Table I. Note that the mathematical model used for

![Overlay of sensorgrams of the binding to PC/PA bilayer for various concentrations of dermaseptin S4. The membrane bilayer, composed of PC/PA (1:1), was immobilized onto an L1 chip. The plot depicts the interaction kinetics for increasing peptide concentrations in PBS at 25 °C. Each sensorgram represents an average curve of two injections.](image)

![Aggregation properties of dermaseptin S4 and derivatives in PBS. Aggregation state is revealed from the concentration dependence light scattering of the peptides. Data represent mean from at least two independent experiments. For aggregating peptides, critical micelle concentration was estimated by extrapolating the curve to the intercept with the x axis as depicted in the inset. Aggregated S4 and monomeric K$_4$S4(1–16)a (asterisks and rectangles, respectively) are shown as typical examples.](image)
the rate constants assumes that the reactants are monomers, whereas aggregation reduces the apparent monomeric concentration by an unknown extent. Therefore, the $K_{app}$ values obtained for aggregated peptides can only be considered indicative. For the sake of comparison, phosphatidylcholine and bovine serum albumin were assayed under the same experimental conditions and found to have affinity constants of $4.2 \times 10^4$ and $<1 \times 10^4$ M$^{-1}$, respectively.

Native dermaseptin displayed a high binding affinity ($3.0 \times 10^6$ M$^{-1}$). Deletion of the eight N-terminal residues resulted in increased binding affinity ($8.2 \times 10^6$ M$^{-1}$). Deletion of both hydrophobic extremities resulted in a binding level below detection, i.e. a loss of binding affinity by more than 3 orders of magnitude ($<1 \times 10^3$ M$^{-1}$). The substituted derivative K$_4$K$_20$-S4 displayed the highest binding affinity ($5.2 \times 10^7$ M$^{-1}$). Compared with K$_4$K$_{20}$-S4, the shorter derivative, K$_4$-S4(1–16)a, displayed a 10-fold reduced binding affinity ($2.9 \times 10^6$ M$^{-1}$). It is noteworthy that truncating the C terminus of the peptide by an additional 3 residues did not affect the binding significantly, but further truncation, to 10 residues, led to loss of binding affinity by 2 orders of magnitude.

Cytotoxicity—To assess the membranolytic potential of the dermaseptin S4 derivatives, their ability to induce hemoglobin leakage was investigated in PBS. In parallel, the ability of the peptides to affect the viability of protozoan cells was investigated in a minimal culture medium to minimize irrelevant peptide interactions with medium components. The resulting leakage was investigated in PBS. In parallel, the ability of the peptides to induce hemoglobin release corresponding to 50% hemolysis at a peptide concentration of 1.5 $\mu$M. An identical peptide concentration was needed to kill 50% of leishmanial cells. However, deletion of the N-terminal hydrophobic domain resulted in a 15-fold reduction of hemolytic activity and a 33-fold reduction of leishmanicidal activity. Truncating both hydrophobic extremities resulted in a general loss of activity. Substitution of positions 4 and 20 by lysines resulted in a 3-fold increase of hemolytic activity, but leishmanicidal activity remained unaffected. Compared with K$_4$K$_{20}$-S4, K$_4$-S4(1–16)a displayed a 30-fold reduction in hemolytic activity but only a 4-fold reduction of leishmanicidal activity. Further C-terminal truncations resulted in further loss of activity with similar proportions. Thus, compared with K$_4$K$_{20}$-S4, K$_4$-S4(1–13)a displayed a reduced hemolytic activity by more than 100-fold but only a 6-fold reduction of leishmanicidal activity. K$_4$-S4(1–10)a was inactive in both assays up to the highest concentration tested (50 $\mu$M).

Susceptibility of cell wall-containing microorganisms (fungi and bacteria) to dermaseptin S4 and its derivatives was assessed by measuring the minimal inhibitory concentration (MIC) of the peptides against three clinical isolates including yeast cells C. neoformans, Gram-positive bacteria S. aureus, and Gram-negative bacteria E. coli. The resulting data are summarized in Table I. Dermaseptin S4 displayed a MIC at the peptide concentration of 4.5 $\mu$M against C. neoformans, and the peptide had a 5-fold reduced potency against Gram-positive bacteria and was virtually inactive against Gram-negative bacteria. Truncation of one or both hydrophobic domains resulted in a general loss of activity. Unlike dermaseptin S4, the derivative K$_4$K$_{20}$-S4 displayed a rather similar potency against the three types of targets (MICs of 2.25, 2, and 1.5 $\mu$M, respectively). The short derivatives, K$_4$-S4(1–16)a and K$_4$-S4(1–13)a, also displayed a nearly homogeneous potency in all three assays with a progressive loss of potency compared with K$_4$K$_{20}$-S4. Finally, K$_4$-S4(1–10)a was weakly active against C. neoformans and E. coli but was virtually inactive against S. aureus up to the highest concentration tested (50 $\mu$M).

**CD Measurements**—Preliminary indications of the peptide structures in solution were obtained with CD measurements in water and in 20% TFE/water. All peptides displayed typical spectra of unordered structure in water (data not shown). In the more hydrophobic medium however, the CD profile of some peptides indicated a clear shift toward an ordered structure, possibly of typical $\alpha$-helix as characterized by double minima at 208 and 220 nm. The CD spectra are summarized in Fig. 3. The structure of native dermaseptin contained $\alpha$-helical components, whereas the CD profiles of S4(9–28) and S4(5–16) progressively shifted toward random structures. The CD profile of K$_4$K$_{20}$-S4 suggested that charge increase led to increased helical content. Interestingly, the data also suggested that the 16-mer and, surprisingly, even the 13-mer derivative maintained a helical structure. The concentration dependence was investigated for K$_4$-S4(1–13)a in the range of 100–400 $\mu$M. No dependence was observed. We cannot exclude the possibility that this is because the lowest concentration tested was already above the concentration at which aggregation is complete. We were unable to obtain unambiguous quantitative data at lower concentrations due to reduced signal strength. The shortest derivative K$_4$-S4(1–10)a displayed random structure.

**NMR Measurements and Resonance Assignment**—Further insight into the molecular organization of the dermaseptin analogs was obtained by conducting NMR structural studies. The proton spectrum of the native S4 peptide in water showed broad unresolved signals indicative of severe aggregation. Furthermore, although various dermaseptin analogs dissolved well in water as shown by the presence of their sharp spectral lines, their two-dimensional NOESY spectra did not contain any NOE characteristics of a defined stable conformation, in agreement with the CD findings (see above). Titrating the aqueous samples with TFE showed that increasing the alcohol content from 0 to 20% v/v induced a marked difference in the appear-
ance of the spectra of K$_4$K$_{20}$-S4, K$_4$-S4(1–16)a and K$_4$-S4(1–13)a substituted analogs (group 1) and S4(9–28), S4(5–16) and K$_4$-S4(1–10)a peptides (group 2). The NOESY spectra of the first group showed numerous strong NOE connectivities suggesting the presence of a stable preferred structure. Further increasing the alcohol content to 100% did not lead to additional changes in their NOESY spectra. On the other hand, the corresponding spectra of the second group were unaffected by addition of TFE indicating that this membrane-mimicking environment does not perturb peptides with no propensity toward helix formation and leaves them in flexible, random coil conformations that are in fast exchange on the time scale of the NMR. The behavior of the S4 peptide was exceptional. Even at high alcohol concentrations, the peptide remained aggregated and therefore was unsuitable for structural studies. Based on these observations, combined with the supporting CD results (see above), an aqueous solution containing 20% v/v trifluoroethanol was chosen for the structural NMR determinations.

The resonance assignments of all dermaseptin analogs were carried out based on the TOCSY and NOESY spectra measured consecutively and under the same experimental conditions, according to the sequential assignment methodology developed by Wüthrich (62). Although no line broadening was detected from the increased viscosity upon addition of TFE (63, 64), strong signal overlap was observed due to the presence of multiple copies of several amino acids, in particular Ala, Lys, and Leu. The weak dispersion of their NH and Hα chemical shifts constituted a major difficulty in peak assignment and necessitated acquiring TOCSY spectra at several temperatures. The line width of the signals remained narrow throughout the measured temperature range (280–345 K), allowing us to assign the vast majority of the signals with the exception of a small number of degenerated γH protons of Lys and Leu (see Table II for the chemical shifts of the first group; chemical shifts of the second group are reported in Supplemental Table I). Moreover, no broadening indicative of aggregation and/or slow exchange was observed for any of the peptides.

Secondary Structure Determination for K$_4$K$_{20}$-S4, K$_4$-S4(1–16)a, and K$_4$-S4(1–13)a—The NOESY spectra of the S4(9–28), S4(5–16), and K$_4$-S4(1–10)a derivatives did not contain any substantial structural information over the entire temperature range in a 20% TFE/water solution. Cooling these samples to 277 K resulted in the appearance of several weak sequential HN-Hα connectivities (data not shown). Their chemical shifts (Supplemental Table I) were consistent with the reported random coil values, and $^{3}$J$_{NH}$-coupling constants ranged between 5.5 and 8.0 Hz indicating an equilibrium average of the different conformers.

Fig. 4, A–C, summarize the NOE cross-peaks detected for the first group of substituted analogs. Analyzing these patterns revealed a remarkable similarity between the spectral features of the N terminus of the K$_4$K$_{20}$-S4 peptide and those of the K$_4$-S4(1–16)a and K$_4$-S4(1–13)a analogs. Almost continuous stretches of $d_{NN(I+1)}$ connectivities were observed between Leu$_2$ and Ala$_{15}$ (K12 for the 13-mer analog), and nearly complete sets of $d_{NN(I+1)}$ and $d_{NN(I+2)}$ cross-peaks were spread throughout each entire peptide. These spectral patterns are typical of an α-helical structure. This finding was further substantiated by the observation of a series of strong NOE cross-peaks of the $d_{NN(I+4)}$ type which are specifically found in α-helices. There were a few resonances that could not be assigned unambiguously (Fig. 4), presumably because of the small dispersion of their chemical shifts due to the α-helix structure. In particular, an aliphatic region of the NOESY spectra showed severe overlap of the resonances, thus preventing unambiguous assignment of the $a(i,i+3)$ pairs which also occur in α-helices. In addition, several $d_{NN(I+2)}$ and $d_{NN(I+3)}$ connectivities, characteristic of a 3$_{10}$ helix, were also identified. In comparison, the NOESY spectrum of the C-terminal part of the K$_4$K$_{20}$-S4 peptide showed only a few, mainly sequential, NOE connectivities that could not be attributed to any defined structural organization. The $^{3}$J$_{NH}$-coupling constants of residues Lys$_{16}$-Ala$_{28}$ varied from 5 to 11 Hz suggesting a conformational dispersion of random coil structures in this region.

The α-helix spanning the N-terminal residues Leu$_2$ to Ala$_{15}$ of K$_4$K$_{20}$-S4 and the entire 16- and 13-mer peptides was confirmed by the small values observed for the $^{3}$J$_{NH}$-coupling constants; most residues had coupling constants below 5 Hz (filled circles in Fig. 4). A few exceptions (open circles) were found within these helical regions, in particular the C terminus of the K$_4$-S4(1–16)a analog, indicating some flexibility at the ends and/or equilibrium between a helical structure and a more random organization.

It is well established that the up-field deviations of the chemical shifts of the αH protons from their random coil values are characteristic of residues adopting an α-helical structure (65, 66). The αH chemical shifts (Table II) of the three analogs were compared with (i) the random coil values in TFE/water solution reported by Merukta et al. (67) and to (ii) the comparative values reviewed by Richards and co-workers (66). Although the latter values were observed for small peptides dissolved in water, it has been shown that random coil αH values are insensitive to the addition of trifluoroethanol up to 30% (68). In Fig. 5 the up-field shifts are summarized for the two short analogs and the N terminus of the longer peptide. The corresponding values of the C-terminal residues of the K$_4$K$_{20}$-S4 peptide did not show any significant structure-related deviations (data not shown). By setting a threshold value of −0.2 ppm from the random coil αH values as indicative of an α-helical structure, it can be seen that the α-helix is confined to the N terminus of the K$_4$K$_{20}$-S4 peptide, i.e., from Ala$_1$ to Lys$_{16}$, and extends over almost the entire K$_4$-S4(1–16)a peptide (except for the values of Leu$_2$, as observed for all three peptides). The magnitudes of the chemical shift deviations found for the majority of the K$_4$-S4(1–16)a residues indicates that even this short peptide adopts a well defined α-helical structure. The deviations of the three C-terminal residues were found to be below the threshold value suggesting conformational flexibility in this region.

NMR-calculated Structures of K$_4$K$_{20}$-S4, K$_4$-S4(1–16)a, and K$_4$-S4(1–13)a—The NMR data collected for the S4(9–28), S4(5–16), and K$_4$-S4(1–10)a peptides (as described above) clearly indicated the lack of any molecular conformation in the 20% TFE/water solution. Molecular modeling of the three-dimensional structures of the K$_4$K$_{20}$-S4, K$_4$-S4(1–16)a, and K$_4$-S4(1–13)a peptides was carried out. The results of the final simulated annealing stage in the XPLOR program for the calculated structures yielded 68, 87, and 60 low energy structures for three peptides, respectively, which had no NOE violation above 0.5 Å, and bond lengths were correct to within 0.05 Å and angles within 5°, and dihedral violations were within 5° of ideal values. These calculated structures were subjected to validation by the Procheck statistical analysis (57). For each peptide, 30 best low-energy structures with residues located only in the “most favored” and “allowed” regions of the Ramachandran plot were chosen for structural analysis. The superpositions of the 15 low-energy calculated structures on their average structure for each peptide are presented in Fig. 6. For the K$_4$K$_{20}$-S4 analog (Fig. 6A), the alignment was optimized for the N-terminal residues Ala$_1$ to Lys$_{16}$, whereas for the other two short analogs (Fig. 6, B and C) all residues were included. As anticipated from the very limited number of NOE
connectivities detected for the C terminus of the K$_4$K$_{20}$-S$_4$ peptide (Fig. 4), the NMR-derived structures showed no convergence in this region of the molecule, confirming the lack of a well defined secondary structure and the inherent flexibility in this region of the molecule. However, the N-terminal segment of the peptide structures lines up into a well defined $\alpha$-helical structure. The r.m.s.d. values calculated between residues 1 and 16 of the 30 low-energy structures and their corresponding average structure are 2.3 Å for the backbone atoms. The structures of the two K$_4$S$_4$(1–16)a and K$_4$S$_4$(1–13)a peptides contain a long $\alpha$-helix spanning the entire length of each peptide with the exception of the terminal residues which typically are more flexible. The r.m.s.d. values, calculated for the entire length of each peptide as above, were 1.9 and 1.3 Å for the backbone atoms, respectively. The fact that the truncated peptides K$_4$S$_4$(1–16)a and K$_4$S$_4$(1–13)a show a defined and stable conformation is unusual considering their short lengths, but the most compelling evidence for the importance of the helical structure conformation of the N termini of the peptides for antimicrobial action is the fact that the first 16 residues of the

| Residue | HN | Hα | Hβ | Others |
|---------|----|----|----|--------|
| Ala$^1$ | 3.98 | 1.35 | \(\gamma\)CH, ND 6CH\_6, 6.84 |
| Leu$^2$ | 8.71 | 4.29 | 1.56 | \(\gamma\)CH, ND 6CH\_6, 6.84 |
| Trp$^3$ | 8.17 | 4.45 | 3.25 | 2H, 7.35 4H, 7.56 5H, 7.03 6H, 7.16 7H, 7.47 eHN, 10.23 |
| Lys$^4$ | 7.58 | 3.80 | 1.58 1.47 | \(\gamma\)CH\_3, 0.91 6CH\_3, 1.37 eCH\_2, 2.79 eHN\_6, ND |
| Thr$^5$ | 7.61 | 4.11 | 3.89 | \(\gamma\)CH\_2, 1.16 |
| Leu$^6$ | 7.76 | 4.11 | 1.58 | \(\gamma\)CH, ND 6CH\_6, 6.84 |
| Ala$^7$ | 8.14 | 4.03 | 1.57 | \(\gamma\)CH, ND 6CH\_6, 6.84 |
| Lys$^8$ | 7.89 | 3.93 | 1.79 | \(\gamma\)CH\_2, ND 6CH\_6, 1.61 1.52 eCH\_2, 2.86 eHN\_6, ND |
| Lys$^9$ | 7.76 | 4.03 | 1.89 1.60 | \(\gamma\)CH\_3, 1.33 6CH\_3, 1.53 eCH\_2, 2.87 eHN\_6, ND |
| Val$^{10}$ | 8.20 | 3.75 | 2.13 | \(\gamma\)CH\_3, 0.86 0.88 |
| Ala$^{11}$ | 8.48 | 4.11 | 1.74 | \(\gamma\)CH, 1.40 6CH\_6, 0.80 |
| Lys$^{12}$ | 8.01 | 4.06 | 1.82 | \(\gamma\)CH\_3, 1.36 6CH\_6, 1.62 1.46 eCH\_2, 2.91 eHN\_6, ND |
| Ala$^{13}$ | 7.92 | 4.18 | 1.39 | |
| Ala$^{14}$ | 8.24 | 4.14 | 1.37 | |
| Ala$^{15}$ | 7.92 | 4.14 | 1.40 | |
| Lys$^{16}$ | 7.96 | 4.17 | 1.83 1.76 | \(\gamma\)CH\_2, ND 6CH\_6, 1.64 eCH\_2, 2.93 eHN\_6, ND |

| Residue | HN | Hα | Hβ | Others |
|---------|----|----|----|--------|
| Ala$^1$ | 4.01 | 1.35 | | |
| Leu$^2$ | 8.71 | 4.29 | 1.58 1.61 | \(\gamma\)CH, ND 6CH\_6, 6.85 |
| Trp$^3$ | 8.17 | 4.45 | 3.25 | 2H, 7.29 4H, 7.47 5H, 7.05 6H, 7.21 7H, 7.48 eHN, 10.20 |
| Lys$^4$ | 7.55 | 3.80 | 1.61 | \(\gamma\)CH\_3, 0.93 6CH\_3, 1.40 eCH\_2, 2.82 eHN\_6, ND |
| Thr$^5$ | 7.61 | 4.11 | 3.93 | \(\gamma\)CH\_3, 1.16 |
| Leu$^6$ | 7.83 | 4.11 | 1.61 | \(\gamma\)CH, 1.63 6CH\_6, 0.82 |
| Ala$^7$ | 8.10 | 4.06 | 1.61 | \(\gamma\)CH, ND 6CH\_6, 0.85 |
| Leu$^8$ | 7.86 | 3.95 | 1.82 1.61 | \(\gamma\)CH\_2, 1.35 6CH\_2, 1.50 eCH\_2, 2.89 eHN\_6, ND |
| Lys$^9$ | 7.79 | 4.09 | 1.89 1.61 | \(\gamma\)CH\_2, 1.34 6CH\_2, 1.51 eCH\_2, 2.91 eHN\_6, ND |
| Val$^{10}$ | 8.16 | 3.82 | 2.13 | \(\delta\)CH\_3, 0.96 0.88 |
| Leu$^{11}$ | 8.36 | 4.19 | 1.71 | \(\gamma\)CH, 1.48 6CH\_6, 0.83 |
| Lys$^{12}$ | 7.98 | 4.16 | 1.84 | \(\gamma\)CH\_3, 1.45 6CH\_3, 1.63 eCH\_2, 2.95 eHN\_6, ND |
| Ala$^{13}$ | 7.98 | 4.16 | 1.39 | |
K4K20-S4 show a well defined helical structure, whereas the C-terminal half of the peptide shows flexibility and no clear structure. Numerous hydrogen bonds between the backbone NH–O atoms were identified in the calculated structures of all three helices. The most abundant donor-acceptor pairs (appearing in over 80% of the calculated structures which fulfilled all the NOE and Ramachandran validation criteria as described above) were found between residues Thr5–Leu2, Leu6–Trp3, Leu7–Lys4, Lys8–Lys4, Lys9–Thr5, Val10–Leu7, and Lys16–Ala14 located at the N terminus of the K4K20-S4 peptide; Leu6–Trp3, Lys8–Lys4, Lys9–Thr5, Leu11–Lys8, and Ala13–Val16 for the K4-S4(1–16)a; and Thr5–Leu2, Leu6–Trp3, Leu7–Lys4, and Lys12–Lys8, for the K4S4(1–13)a analog.

Spatial Distribution of the Electrostatic Potential for K4K20-S4 (A) and K4-S4(1–16)a (B) was aligned over residues Ala1−Lys16 at the N terminus. Peptides K4-S4(1–16)a (B) and K4-S4(1–13)a (C) were superimposition in their entirety.

Summary of the NOE connectivities observed for K4K20-S4 (A), K4-S4(1–16)a (B), and K4-S4(1–13)a (C) in 20% TFE/water. Coupling constants are indicated with filled circles for $^{3}J_{NH} < 5$ Hz, open circles for $5 < ^{3}J_{NH} < 7.5$ Hz, and filled squares for $^ {3}J_{NH} > 8$ Hz. Residues Ala18 (in A) and Lys16 (B) could not be fitted due to overlap.
structures of the three peptides has a strong inclination to fold in view of the environment represented by 20% TFE/water. Folding enabled the topological segregation of charged residues from uncharged ones, thus forming a bi-lobed amphipathic structure that, besides its inherent hydrophobic/hydrophilic characteristics, engendered a positive electrostatic potential that extended well beyond the physical dimensions of the peptides. The fact that this potential distribution is common to all the active peptides indicates a role in interactions of the cytolytic peptide with target membranes.

Native dermaseptin S4 displayed highly lipophilic properties as suggested by its high $K_{\text{app}}$ to the model membrane and as indicated by its tendency to aggregate in solution (NMR and light scattering data). Furthermore, dermaseptin S4 displayed high membranolytic activity against red blood cells and Leishmania, with very rapid kinetics (lysis of both types of these cells can be observed under microscope within seconds, data not shown). These properties may be rationalized with respect to the steps involved in the theoretical mechanism of action where monomers are stipulated to adhere to the membrane until reaching a threshold concentration. Our data suggest that aggregation is responsible for the rapid and highly efficient activity because naked membranes are contacted by a preformed active entity. Therefore, the relative inactivity of such an entity against E. coli may be directly related to the inability of the peptide to cross the external membrane as supported by the progressive loss of activity with increasingly impermeable cell walls (C. neoformans > S. aureus > E. coli). The fact that aggregated peptides become fully active against E. coli if tested in the presence of EDTA further supports this possibility.2

Aggregation in solution and its consequences could affect the

**DISCUSSION**

Toward better defining the factors affecting selective cytotoxicity, we investigated in the present study the relation between peptide molecular organization in solution upon encountering its target and peptide cytolytic effect, taking into account the ability of the peptide to access the target membrane. We first tried to understand how physicochemical parameters affected binding to an idealized charged membrane model. Then peptide properties were correlated with their capacity to affect cells whose plasma membrane is readily accessible (erythrocytes and protozoa), as well as cells for which accessibility is hampered due to the presence of surrounding cell walls (fungi and bacteria). Extensive NMR data were taken for all dermaseptin peptides under investigation. The ensembles of the structured peptides and peptide regions composed a large number of structures and showed good convergence with low r.m.s.d. values clearly indicating the presence of high resolution, well defined, preferred helical structures at the N terminus of K$_4$K$_{20}$-S4, and for the entire K$_4$-S4(1–16)a and K$_4$-S4(1–13)a peptides. The Procheck statistical evaluations completely corroborated these findings. The data provided clear information regarding the conformational state of the S4 analogs constituting a solid basis for analyzing the chemical-physical properties of these peptides and allowing us to address certain aspects of the mechanism of action.

NMR-derived structures enabled the delineation of structural characteristics that we believe form the basis for the activity of dermaseptin S4 and, perhaps, of many other antimicrobial peptides to an extent. From a purely structural point of view, the data revealed the fact that the amino acid sequence common to the three peptides has a strong inclination to fold in the environment represented by 20% TFE/water. Folding enabled the topological segregation of charged residues from uncharged ones, thus forming a bi-lobed amphipathic structure that, besides its inherent hydrophobic/hydrophilic characteristics, engendered a positive electrostatic potential that extended well beyond the physical dimensions of the peptides. The fact that this potential distribution is common to all the active peptides indicates a role in interactions of the cytolytic peptide with target membranes.

Native dermaseptin S4 displayed highly lipophilic properties as suggested by its high $K_{\text{app}}$ to the model membrane and as indicated by its tendency to aggregate in solution (NMR and light scattering data). Furthermore, dermaseptin S4 displayed high membranolytic activity against red blood cells and Leishmania, with very rapid kinetics (lysis of both types of these cells can be observed under microscope within seconds, data not shown). These properties may be rationalized with respect to the steps involved in the theoretical mechanism of action where monomers are stipulated to adhere to the membrane until reaching a threshold concentration. Our data suggest that aggregation is responsible for the rapid and highly efficient activity because naked membranes are contacted by a preformed active entity. Therefore, the relative inactivity of such an entity against E. coli may be directly related to the inability of the peptide to cross the external membrane as supported by the progressive loss of activity with increasingly impermeable cell walls (C. neoformans > S. aureus > E. coli). The fact that aggregated peptides become fully active against E. coli if tested in the presence of EDTA further supports this possibility.2

Aggregation in solution and its consequences could affect the
activity of many other antimicrobial peptides in a similar fashion. For instance, the bee venom peptide, melittin (69), or the human peptide, LL-37 (70), were reported to form aggregates in aqueous solutions. Both peptides are highly hemolytic, whereas their non-aggregated derivatives display significantly reduced hemolytic activity (70). Surprisingly, deleting the eight N-terminal residues of S4 resulted in increased binding affinity. Although this could reflect inaccurate determination of the affinity constant of dermaseptin S4 (because of its aggregated state), it could also mean that monomeric S4(9–28) is genuinely endowed with increased binding properties. This possibility is supported by the fact that S4(9–28) displayed a lower dissociation constant (data not shown). Regardless, although S4(9–28) was endowed with high binding affinity, the peptide was inefficient in affecting cell viability, and both NMR and CD data showed its low tendency toward folding in solution. This indicates that binding does not require particular folding and is required for but does not necessarily lead to membrane disruption. To induce membrane disruption the peptide needed the properties encompassed by the missing N-terminal sequence, including the ability to fold properly. It is therefore not surprising that truncating both hydrophobic domains resulted in dramatic loss of structure, binding, and all activities assessed even though the original net charge of +4 was preserved (Table I).

K$_{K20}$S4 also exhibited high membranolytic activity against red blood cells and Leishmania. This correlated nicely with the increased binding affinity of the peptide compared with dermaseptin S4. Although the NMR structure of S4 was not resolved due to its high aggregation state, it is possible that the increase in potency is also related to a quantitative increase in the electrostatic potential which in turn could be responsible for enhanced binding due to the enlarged charged surface displayed by K$_{K20}$S4. This possibility will hopefully be elucidated in future studies. In contrast, K$_{K20}$S4 was about 2-fold less aggregated in PBS and displayed nearly equipotent cytotoxicity against all types of cells tested. The following interpretation can account for our observations. If we consider the peptide solution under monomer/oligomer equilibrium, the data can be interpreted as follows: twice as many molecules of K$_{K20}$S4 are present as monomers in the solution compared with S4. If there are enough monomers in the solution (note that estimated CMC is approaching the average MIC), then the cell wall containing pathogens could become permeable to monomeric K$_{K20}$S4, i.e., the peptide will be able to reach the plasma membrane and exert its membranolytic potential that is increased due to its higher charge.

To obtain a quantitative estimation of monomer units ($n$) within the aggregate, an attempt to determine $n$ for S4 and K$_{K20}$S4 was made using SDS-PAGE as described previously (71). However, both peptides gave smears of molecular weight that increased with increasing peptide concentration. The range of molecular mass was estimated between 6 and 21 kDa for the highest concentration tested (350 µM). Under these conditions $n$ may be estimated as being between 2 and 6.

The high binding affinity displayed by S4(9–28) suggested that binding affinity is mostly influenced by the C-terminal sequence. Yet, truncation of the C-terminal sequence did not abolish binding. This reflects the contribution of the N-terminal sequence to binding, particularly because its net charge content, was increased (from +4 to +6 in K$_{20}$S4(1–16a), which seems to have greatly compensated for the truncated C terminus. Moreover, the reduced binding affinity displayed by K$_{20}$S4(1–16a) compared with K$_{K20}$S4 correlated nicely with the loss of hemolytic and anti-leishmanial activities but contrasted the fact that K$_{20}$S4(1–16a) hardly lost any potency against yeast and bacteria. This may be rationalized in light of the aggregation and structural results. Because the peptide is no longer aggregated in solution, its activity can be expected to follow the experimental “rule” that endows potent membranolytic properties to highly charged peptides that have the ability to fold into an amphipathic structure. Our structural data suggest that when K$_{20}$S4(1–16a) enters into an interaction with the target membrane, the peptide presents a structure similar to the amphipathic structure of K$_{K20}$S4, although more compact and with modified hydrophobic properties (due to the truncated C terminus). Accordingly, monomeric K$_{20}$S4(1–16a) exerts more potency on membranes of microorganisms than on red blood cell membranes for the “classical” (and yet poorly understood) reasons related for instance to the presence of a transmembrane potential, negative charge density, and membrane fluidity.

Similar observations can be made regarding K$_{20}$S4(1–13a). Compared with K$_{20}$S4(1–16a), the shorter derivative was only slightly less active despite having lost both charge and hydrophobicity. Its preserved activity seems to result from maintaining the same three-dimensional structure and a similar electrostatic potential. As for K$_{20}$S4(1–10a), having further lost both charge and hydrophobicity in addition to having lost the ability to fold into an amphipathic structure, the peptide became incapable of interacting properly with membranes and efficiently affecting target cell integrity.

The biological and structural data are well correlated. The N-terminal regions of all active, structured S4 analogs have similar three-dimensional features that govern the spatial distribution of the electrostatic potential. Our findings indicate that this property is necessary for cytotoxicity and without it the activity is severely curtailed; S4(5–16) has all the four lysine residues of active S4 and the same net positive charge as active K$_{20}$S4(1–13a), but because it is unstructured it is inactive. K$_{20}$S4(1–10a), lacking one positively charged residue, loses its ability to maintain the bi-lobal potential distribution leading to a severe reduction in activity. The extent of this positive electrostatic lobe correlates the degree of cytolytic activity as seen in the increase of activity between K$_{20}$S4(1–13a) and K$_{20}$S4(1–16a). Another essential factor is the ability of the peptide to bind to the target membrane. Our studies show that the C terminus enhances binding properties. The S4(9–28)-truncated analog represents the highly hydrophobic C-terminal region of S4 and shows the strongest binding affinity but is unstructured and has no activity. The combined effect of these two characteristics, the extended positive electrostatic potential and the binding affinity of the hydrophobic C terminus, is essential for efficient cytolytic activity. K$_{K20}$S4 is the most potent of the studied peptides as it incorporates both these features. In conclusion, the data presented support the idea that cytotoxic activity of dermaseptins, and possibly other antimicrobial peptides, arises from the generation of a bi-lobed electrostatic potential field. Activity is expected to persist as long as this amphipathic organization is maintained. Whereas potency is a direct function of this electrostatic potential field, selectivity (the spectrum of action) is affected primarily by the hydrophobic properties of the peptides. The usefulness of our approach for the design of selective cytolytic peptides is demonstrated by our having transformed the native dermaseptin S4 from a weak antibacterial and highly hemolytic peptide to a range of peptides that exert enhanced antibacterial activity but reduced hemolysis. Such peptides could be useful in various applications in the antimicrobial field.

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