Solution Structure of Spheniscin, a β-Defensin from the Penguin Stomach* [S]

Céline Landon‡, Cécile Thouzeau§, Henri Labbé††, Philippe Bulet‡, and Françoise Vovelle**

From the ‡Centre de Biophysique Moléculaire, CNRS UPR 4301, Université d'Orléans, rue Charles Sadron, 45071 Orléans Cedex 2, France, the §Centre d'Ecologie et de Physiologie Energétiques, CNRS UPR 9010, 23 rue Becquerel, 67087 Strasbourg Cedex 02, France, and ††Atheris Laboratories, Case Postale 314, CH-1233 Bernex, Geneva, Switzerland

Recently two β-defensins, named spheniscins, have been isolated from the stomach content of the king penguin (Aptenodytes patagonicus), which is capable of preserving food for several weeks during egg incubation (Thouzeau, C., Le Maho, Y., Froget, G., Sabatier, L., Le Bohec, C., Hoffmann, J. A., and Bulet, P. (2003) J. Biol. Chem. 278, 51053–51058). It has been proposed that, in combination with other antimicrobial peptides, spheniscins may be involved in this long term preservation of food in the bird's stomach. To draw some structure/function features, the three-dimensional structure in aqueous solution of the most abundant spheniscin (Sphe-2) was determined by two-dimensional NMR and molecular modeling techniques. The overall fold of Sphe-2 includes a three-stranded antiparallel β-sheet stabilized by three disulfide bridges with a pairing typical of β-defensins. In addition, the N-terminal segment shows helical features on most structures. Sphe-2 is highly cationic, and its surface displays a hydrophobic patch. Comparative modeling revealed that this patch is preserved in avian defensins. The activity of Sphe-2 against a pathogenic Gram-positive strain was retained in vitro in the conditions of osmolarity found in penguin stomach content and also in different salt concentrations.

During the final stage of egg incubation in king penguins (Aptenodytes patagonicus), the male can preserve undigested food in the stomach for several weeks (1). This ensures survival of the newly hatched chick in the event that the return of the foraging female from the sea is delayed. In accordance with the characterization of stress-induced bacteria (2), a previous study has demonstrated that numerous antimicrobial activities exist in preserved stomach contents (3). Two antimicrobial peptides have been isolated and fully characterized, namely spheniscin-1 and -2 (Sphe-1/pBD-1 and Sphe-2/pBD-2). The two forms of spheniscins differ by a single residue, His1, in Sphe-1 versus Arg14 in Sphe-2. A data bank search revealed that spheniscins are members of the well known defensin family.

Defensins are small (3–5 kDa) cationic antimicrobial peptides that are part of the innate immunity of vertebrates (4, 5), invertebrates (6), and plants (7). In vertebrates, defensins can be divided into α-, β-, and ω-defensin subfamilies. The unusual ω-defensin, which is a circular peptide, was initially isolated from rhesus macaque (8). Recently a pseudogene coding for a homologue, named retrocyclin, was found in the human genome (9). The α- and β-defensins are the most widespread in nature, and they are subclassified according to precursor and cysteine residues of their sequence. In α-defensins, the disulfide pairing is Cys1-Cys6, Cys2-Cys4, and Cys3-Cys5, whereas it is Cys1-Cys5, Cys2-Cys4, and Cys3-Cys6 in β-defensins. In spheniscins, the disulfide pattern is identical to that of β-defensins (3). β-Defensins are more widely distributed than α-defensins as only β-defensins have been isolated from birds. In addition to the two spheniscins isolated from the king penguin, bird β-defensins have been found in polymorphonucleated granulocytes and in epithelia of chickens and turkeys. Four β-defensins named gallinacins and chicken heterochrom peptide (Gall-1/CHP1, Gall-1a/CHP2, Gall-2, and Gall-3) have been reported in Gallus gallus (10–12), and four have been reported in the turkey Meleagris gallopavo, named gallopavin-1 (GPV-1) and THP-1, THP-2, and THP-3 for turkey heterochrom peptide 1–3 (11, 12). Sphe-1 and -2 have been isolated from the stomach contents of king penguins that efficiently conserve food for several weeks (3).

The activity of a synthetic version of Sphe-2 is preferentially...
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directed against Gram-positive bacteria including pathogenic strains and is of bactericidal type. Interestingly, when an activity was recorded against Gram-negative strains, the effect of Sphe-2 is mainly of bacteriostatic type. This contrasts with the activity generally observed for β-defensins as most of them are effective against Gram-negative bacteria and yeast cells. In fact, the activity of spheniscin is closer to that of α-defensins that are microbicidal against Gram-positive and Gram-negative bacteria, yeast fungi, and several enveloped viruses (4).

This biological property of the spheniscins compared with other vertebrate β-defensins in addition to (i) the low percentage of similarity with mammalian β-defensins, (ii) their high efficacy on human pathogenic fungi, and (iii) their location within the rather unfavorable environment of the penguin stomach prompted us to investigate the three-dimensional structure of Sphe-2. The structure of six β-defensins has been solved by NMR or x-ray crystallography (13–19): human β-defensins HBD-1, HBD-2, and HBD-3; murine β-defensins mBD-7 and mBD-8; and bovine neutrophil β-defensin-12 (BNBD-12), but no crystal or NMR solution structure of a bird β-defensin is available.

In this study, we determined the three-dimensional structure of the king penguin β-defensin, Sphe-2, in aqueous solution by two-dimensional 1H NMR spectroscopy and molecular modeling and compared this structure to those of the closest β-defensins from mammals. The global fold of Sphe-2, as of most β-defensins, includes a well-defined three-stranded β-sheet and a short N-terminal domain with an α-helical propensity. However, differences were observed in the distribution of the charged and hydrophobic residues. While HBD-2 and BNBD-12 are amphipathic, HBD-3 is mainly hydrophilic. Sphe-2, although very cationic, is slightly less hydrophilic than HBD-3, and its surface displays a small hydrophobic patch that was not described in HBD-3 but apparently preserved in avian HBD-3, and its surface displays a small hydrophobic patch that was not described in HBD-3 but apparently preserved in avian

EXPERIMENTAL PROCEDURES

Peptide Synthesis and Purification—Synthetic Sphe-2 was obtained from Altegen Laboratory (Schtigheim, France). The integrity, purity, and correct refolding were confirmed by MALDI-TOF-MS fingerprinting of a tryptic digest of 2 μg of peptide following the experimental procedure reported previously (3). Briefly purified Sphe-2 (native and synthetic) was treated with trypsin (Roche Applied Science) at an enzyme/substrate ratio of 1:10 (w/w), and the digest was analyzed by MALDI-TOF-MS using α-cyano-4-hydroxycinnamic acid as matrix.

NMR Experiments—The NMR sample was prepared by dissolving 5.5 mg of synthetic Sphe-2 in 600 μl of H2O/D2O (90:10) to obtain a final concentration of 2 mM. All 1H NMR spectra were recorded on a Varian INOVA NMR spectrometer equipped with a z-axis field-gradient unit and operating at a proton frequency of 600 MHz. The pH was adjusted to 4.3, and the double quantum filtered correlation spectroscopy (20), clean TOCSY (21), and NOESY (22) experiments were performed at 293K. Clean TOCSY was acquired using a mixing time of 80 ms; NOESY spectra were performed with mixing times of 120, 160, and 300 ms. All spectra were referenced to the residual H2O signal set as the carrier frequency, 4.821 ppm at 293 K, and were processed with NMRPipe/NMRDraw softwares (23). The identification of amino acid spin systems and the sequential assignment were performed using the standard strategy described by Wuthrich (24). To identify the exchange rate of backbone amide protons, the sample was lyophilized and quickly (25). Since the disulfide pairing is known (3), covalent bonds were built between the sulfur atoms of the paired cysteine residues, Cys2-Cys3, Cys2-Cys5, and Cys17-Cys18. Structure calculations were carried out with ARIA 1.1 (26) implemented in the software CNS 1.1 (27). ARIA is a powerful approach using an iterative process to perform the assignment of ambiguous NOEs from structures calculated using a combination of NOEs assigned at the previous step and a set of ambiguous distance restraints treated as the sum of contributions from all possible assignments. Each ARIA run included nine iterations. In the last step, the 20 best structures were refined by molecular dynamics in explicit solvent to remove artifacts due to the simplification of the force field used in the previous steps. A table of chemical shifts and a first set of distance restraints deduced from easily assigned NOEs were introduced as input to ARIA, and the calculations were initiated. The “ambicutoff” (26) for accepting or rejecting possible assignments was modified compared with the default values. In the standard ARIA protocol, p varies from 0.999 to 0.8 during the whole process. The variation was reduced from 0.999 to 0.9 to prevent the elimination of weak contributions to ambiguous cross-peaks that are essential for the formation of the secondary structure elements. At the end of each run, the new assignments proposed by ARIA were checked manually and introduced (or not) in the following run. Rejected restraints and residual violations were also analyzed, and assignments were corrected if required. This iterative process was repeated until complete assignment of the NOE map was achieved. A last run of 100 structures was then performed with the final list of NOE-derived distance restraints in which no restraint can be rejected. The 10 structures with the lowest energy were considered as characteristic of the peptide structure.

The structures were displayed and analyzed using the MOLMOL (28) and SYBYL (SYBYL85.5, TRIPOS Inc., St. Louis, MO) programs, and their quality was evaluated using the PROCHECK (29) and PROSMART (30) softwares. The formation of hydrogen bonds was established according to distance criteria. Lipophilic and electrostatic potentials were calculated and represented using the MOLCAD option of SYBYL.

Salt Effect on the Antibacterial Activity of Sphe-2—The effect of sodium and magnesium chloride on the antibacterial activity of Sphe-2 was assessed using a liquid growth inhibition assay (31). Growth inhibition was estimated by measurement of the minimal inhibitory concentration (MIC) against selected strains, the Gram-negative Escherichia coli and the pathogenic Gram-positive Staphylococcus aureus. Briefly logarithmic phase bacterial cultures were diluted to an A600 of 0.001 (approximately 108 colony-forming units/ml) in a broth supplemented or not with various concentration of salts. Diluted bacteria (90 μl) were mixed with 10 μl of either distilled water (control) or different concentrations of Sphe-2 ranging from 0.75 up to 100 μM. Bacterial growth was measured after an overnight incubation at 30 °C by measuring the change in the absorbance of the culture at 600 nm with a microplate reader. The MIC value corresponds to the interval of concentration [a] – [b] where [a] is the highest concentration tested at which the bacteria are growing and [b] is the lowest concentration that causes 100% inhibitory growth (32). To assess the salt effect on the bacterial growth, the poor broth medium (1% bacitracyn, 85 mM NaCl, pH 7.4) was supplemented with NaCl and MgCl2 separately or in combination. The following final salt concentrations were assayed: (i) 180 and 480 mM in NaCl, (ii) 1 and 50 mM in MgCl2, and (iii) 160 mM NaCl plus 1 mM MgCl2. Osморality of the different media and of the stomach content was determined using an automatic milliosmometer (type 13/13DR, Roehling, Berlin, Germany).

RESULTS

NMR Data—After identification of the spin systems on the correlation spectroscopy and TOCSY spectra, sequence-specific assignments were obtained from the sequential connectivities observed on the amide-α, amide-amide cross-peak region of the NOESY spectrum. The fingerprint region of the TOCSY and NOESY spectra are shown in Fig. 1 (see the complete maps in the supporting information). All protons were assigned except for the amide proton of Phe8, which was not observed in our experimental conditions; the arginine ϵ NH2; the serine OH; and the N-terminal NH3+ groups, which are in very fast exchange with water. In addition, the ξ protons of Phe residues could not be assigned unambiguously due to overlapping with the other aromatic protons (see the chemical shift table in the supporting information).

Low field shifted Hα shifts and a characteristic set of interstrand Hα-Hα, NH(i)-NH(j), and Hα(i)-NH(j)
connectivities delineate a triple-stranded $\beta$-sheet. The presence of medium range NOEs in the rest of the sequence suggests that it consists mainly of loops and turns. Strong sequential Hα/Hβ NOE cross-peaks for Xaa-Pro peptide bonds indicate a trans configuration for Pro 20 and Pro 23.

Twenty-one NH signals, exchanged before the acquisition of the first one-dimensional spectra ($t = 5$ min), are described as “very fast exchanging protons.” Four additional amide proton signals disappeared within the first 18 h. The residual amide protons, not exchanged after 18 h, are all located in the $\beta$-sheet (Phe 11, Ala 13, Ile 22, Ile 24, Cys 27, Gln 32, Cys 33, Cys 34, and Arg 35) except for the NH of Gly 10 and Val 31 (Fig. 1) (see the short TOCSY spectra recorded in D$_2$O to monitor the exchange data in the supporting information).

**Structure Calculations**—The NOE data set used in the final ARIA run included 594 distance restraints (Table I) involving 335.2 intraresidue, 139.7 sequential, 32 medium range ($2 \leq |i - j| \leq 4$), and 87.1 long range ($|i - j| \geq 5$) restraints with an average of 16 restraints/residue. Among these restraints, 551 are non-ambiguous. Ten structures, in very good agreement with all the experimental data and the standard covalent geometry, were selected for further analysis. For these structures, no experimental distance constraint violation greater than 0.3 Å was observed and the root mean square deviation (r.m.s.d.) values, with respect to the standard geometry, are low. The Ramachandran plot exhibits 95% of the $\phi$, $\psi$ angles of the 10 converged structures in the most favored and additional allowed regions according to the PROCHECK software nomenclature. The selected structures display small potential energy values. In particular negative van der Waals and electrostatic energy values are indicative of favorable non-bonded interactions.

**Structure Description**—The overall fold of Sphe-2 is typical of $\beta$-defensins including a twisted three-stranded antiparallel $\beta$-sheet with a $(+2X, -1)$ topology as defined by Richardson (33). Strand $\beta_1$ (Phe$^{11}$-Ala$^{13}$) is hydrogen-bonded to strand $\beta_3$ (Val$^{31}$-Arg$^{35}$), which in turn is hydrogen-bonded to strand $\beta_2$ (Ser$^{21}$-Cys$^{27}$) (Fig. 2). Residues Ile$^{24}$ and Gly$^{25}$ are both hydrogen-bonded to Cys$^{33}$ (Fig. 1) leading to a $\beta$-bulge in $\beta_2$. The amide protons of all residues involved in hydrogen bonds exhibited a slow exchange rate in D$_2$O (Fig. 1), and the two other residues with a slow NH exchange rate, Gly$^{10}$ and Val$^{31}$, are embedded in the structure and particularly inaccessible to the solvent. Strands $\beta_1$ and $\beta_2$ are separated by a long loop, L1, including a type IV $\beta$-turn between residues Arg$^{19}$ and Ser$^{21}$ and a type I $\beta$-turn involving residues Ser$^{24}$ to Val$^{31}$. Due to a low number of NOEs in this region, the N-terminal segment (Ser$^1$-Gly$^{10}$) is less “well” defined (Fig. 2). In fact, a small
helical turn is formed on a majority of structures, but depending on the structure it was considered by PROMOTIF as an α-helix or as a succession of turns. In addition, a PROCHECK analysis showed that most residues in the segment from Phe 2 to Arg 8 lie in the helical region of the Ramachandran plot.

As evidenced by the r.m.s.d. calculations (Table I) and by a superposition of the backbone of the selected structures (Fig. 2), the β-sheet region of Sphe-2 is well defined with a pairwise r.m.s.d. on the Ca atoms of 0.54 Å. The pairwise r.m.s.d. calculated for all Ca backbone atoms is large (1.93 Å) due to the N-terminal segment (2.40 Å) and to the L1 loop (1.55 Å).

The tertiary structure of Sphe-2 is very compact. The hydrophobic residues are scattered all over the rest of the molecule and are separated by hydrophilic residues. With 10 arginine residues over 38 amino acids and no anionic residue, Sphe-2 is a highly cationic molecule. The charges are spread all over the surface of the molecule, and the electrostatic potential calculated with SYBYL using Kollman charges (34) varied between −15 and +380 kcal mol⁻¹ so that the surface of the molecule is almost entirely positively charged except for the C terminus (Fig. 3A).

**DISCUSSION**

We determined the three-dimensional structure of Sphe-2 in aqueous solution. Sphe-2 is a β-defensin isolated from the stomach content of three different penguins (324 mosM) and to the osmolarity of the plasma of seawater fishes (337 mosM) (35). When the concentration in sodium chloride was increased up to 480 mM, the efficacy of Sphe-2 against S. aureus decreased by a factor of 16 (MIC, 50–100 μM). In the control experiment with poor broth supplemented at 480 mM NaCl, E. coli did not grow properly.

In the presence of 1 mM MgCl₂, the activity of Sphe-2 against S. aureus was unchanged, while at 50 mM a decrease by a factor of 8 was observed. In the presence of 1 and 50 mM of MgCl₂, the growth of E. coli was altered by factors of 2 and 4, respectively. Combining monovalent ions (160 mM NaCl) and divalent ions (1 mM MgCl₂) in the range of the values found in seawater fishes (348 mosM) (35). When the concentration in sodium chloride was increased up to 480 mM, the efficacy of Sphe-2 against S. aureus decreased by a factor of 16 (MIC, 50–100 μM). In the control experiment with poor broth supplemented at 480 mM NaCl, E. coli did not grow properly.

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**Salt Sensitivity of Sphe-2**—In control poor broth medium (85 mM NaCl, 216 mosM), Sphe-2 is active at a concentration below 6 μM against the two microorganisms selected for this study, S. aureus (Gram-positive) and E. coli (Gram-negative) (Table II). An increase in the NaCl content to a concentration of 160 mM, representing an intermediate concentration between the values observed in seawater fishes and mammals (35), did not affect Sphe-2 activity. The osmolarity measured for this culture medium (348 mosM) was close to the value measured in the stomach content of three different penguins (324 ± 23 mosM) and to the osmolarity of the plasma of sea water fishes (337 mosM) (35). When the concentration in sodium chloride was increased up to 480 mM, the efficacy of Sphe-2 against S. aureus decreased by a factor of 16 (MIC, 50–100 μM). In the control experiment with poor broth supplemented at 480 mM NaCl, E. coli did not grow properly.

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**DISCUSSION**

We determined the three-dimensional structure of Sphe-2 in aqueous solution. Sphe-2 is a β-defensin isolated from the stomach contents of the king penguin and shows a wide array of antimicrobial activity against Gram-positive and Gram-negative bacteria, yeasts, and fungi (3). It displays all the structural characteristics of mammalian β-defensins, including a common array of disulfide bridges and the presence of a three-stranded β-sheet as the main secondary structure element. The
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Fig. 3. Global fold and lipophilic and electrostatic potentials of Sphe-2 (A), HBD-3 (B), and HBD-2 (C). Left column, schematic representation of the backbone. Central column (panels 2 and 3), hydrophobic and hydrophilic potential areas, calculated with the MOLCAD option of SYBYL at the Connolly surfaces, are displayed in brown and blue, respectively. Green surfaces represent an intermediate hydrophobicity. A common scale is used for the three molecules. The orientation of panel 2 is given by the backbone representation. A 180° rotation with respect to a vertical axis is applied between panels 2 and 3. Right column (panels 4 and 5), electrostatic positive and negative areas are in red and blue, respectively. Intermediate areas are in green. A common scale is used for the three molecules (+300, −300 kcal mol⁻¹). The orientation of panel 4 is given by the backbone representation (left column). A 180° rotation with respect to a vertical axis is applied between panels 4 and 5. term, terminus.

Table II  
Effect of sodium and magnesium chloride on Sphe-2 activity

| Final salt concentration in poor broth | Osmolarity | MIC       |
|--------------------------------------|------------|-----------|
| 85 mM NaCl                           | 216        | 3–6       |
| 160 mM NaCl                          | 348        | 3–6       |
| 480 mM NaCl                          | 961        | 30–100    |
| 85 mM NaCl + 1 mM MgCl₂             | 220        | 3–6       |
| 85 mM NaCl + 50 mM MgCl₂            | 344        | 25–50     |
| 160 mM NaCl + 1 mM MgCl₂            | 352        | 3–6–12    |

| S. aureus | E. coli |
|-----------|---------|
| 1.5–3.0   | 6–12    |

*Control poor broth.  
*The bacterial strain did not grow properly in the control experiment.  
*Abnormal bacterial growth, characterized by the presence of a bacterial pellet, was present.

The second strand of the β-sheet includes a bulge found in all α- and β-defensins of known structures. Despite a lack of NOEs in the N-terminal segment, a small helix or a short segment with helical features is found on most structures. Such a small helix or a helical turn, including the first cysteine residue, is observed in mammal β-defensins but not in α-defensins. The only exception is the β-defensin from bovine neutrophils, BNBD-12 (19), which displays a rather disordered N-terminal strand.

In addition to Sphe-2, a series of β-defensins has been isolated from chickens and turkey (10–12). The sequences of these bird defensins are aligned with Sphe-2 in Fig. 4. Gal-3 from the chicken G. gallus and GPV-1 from the turkey M. gallopavo (12) share the strongest percentage of similarities to Sphe-2 with 50 and 47% of identity, respectively. The percentage of identity to Gal-1/CHP1 and Gal-1α/CHP2 from chicken and to THP-1 and THP-2 from turkey (10, 11) is slightly lower (37–39%). Finally, Gal-2 is more distant from Sphe-2 with 33% of identity. Besides the cysteine disulfide array, several residues are strictly conserved between Sphe-2 and the other avian β-defensins: a basic residue (Arg or Lys) at position 8 (Sphe-2 numbering), a glycine residue at position 10, a hydrophobic residue (Ile, Leu, or Val) at position 22, a glycine residue at position 25, a phenylalanine at position 30, and finally two hydrophobic residues at the C terminus. Among the four Gly-Xaa-Cys motifs observed in the Sphe-2 sequence, two are also conserved in the other avian β-defensins, one at the beginning of β1 (with glycine at position 10) and the second in β2 (with glycine at position 25). The only exception is GPV-1 where the second Gly-Xaa-Cys motif is replaced by an Ala-Xaa-Cys motif.

There was no previous structure available for bird defensins. However, assuming that other avian β-defensins adopt a structure similar to Sphe-2, we built structural models with MODELLER 6.0 (36) using the Sphe-2 structure as template (data not shown). We noticed that the hydrophobic patch observed on Sphe-2 surface (Fig. 3A) is well but not strictly preserved in most other avian defensins. In particular, the replacement of the doublet Phe19-Pro20 of Sphe-2 by the sequence Ser-His in THP-2 and Gal-2 considerably reduces the hydrophobicity of this region. Another important feature concerns the electrostatic properties of the molecules. Even if all other avian β-defensins are cationic, they are less positively charged than Sphe-2 (global charge, +10), and the positions of charged residues are rather variable. The differences observed in the hydrophobic and electrostatic properties of bird defensins are probably responsible for differences in the activity spectra of these molecules. However, the activity spectrum of bird defensins is poorly documented.
are no published data for Gal-3 and GPV-1 and only partial information on the antimicrobial activity of the others (10, 11, 37). Until more details become available, it will be particularly difficult to draw structure/activity relationships between Sphe-2 and the other avian β-defensins.

To investigate structure/activity relationships, we therefore searched in the Protein Data Bank (PDB) (38) for structural neighbors of Sphe-2 using DALI (39). Among the eight defensins (two α and six β) available in the PDB, the best scores were obtained for three β-defensins, two human defensins, HBD-2 (PDB code 1fd3) and HBD-3 (PDB code 1kj6), and the bovine neutrophil defensin BNBD-12 (PDB code 1nbm). Interestingly a good score was also obtained for the α-defensin from rabbit kidney RK-1 (PDB code 1ews). Although structurally very close to Sphe-2, with a r.m.s.d. of 1.31 Å between the Ca atoms of aligned residues (Table III), RK-1 displays only 28% of identity to Sphe-2 (Fig. 4B). The distribution of residues at the surface of Sphe-2 and RK-1 are very different (data not shown). The hydrophobic patch is not conserved, and with a global charge of +1, RK-1 is not really cationic. RK-1 is active against *E. coli* at rather high concentrations (15–150 μg/ml) but can also activate Ca2+ channels in vitro (40). This means that, in addition to being antibacterial, it bears some toxin properties. The bovine β-defensin BNBD-12 displays 37% of sequence identity to Sphe-2 (Fig. 4B), and the r.m.s.d. is 1.33 Å for 28 aligned residues (Table III). BNBD-12 is active against *E. coli* and *S. aureus* at concentrations of the micromolar range (41). The human β-defensin HBD-2, although structurally related to Sphe-2 with a r.m.s.d. of 1.25 Å for 28 aligned residues (Table III), exhibits only a little sequence identity with Sphe-2 (18%) (Fig. 4B). In terms of efficacy, as far as the activity data can be compared, HBD-2 is less potent than Sphe-2 against Gram-negative (*E. coli* and *Pseudomonas aeruginosa*) and Gram-positive (*S. aureus*) bacteria but more active against the yeast *Candida albicans*. Interestingly, regarding the killing activity, Sphe-2 is a bactericide against *S. aureus*, while HBD-2 is only bacteriostatic (3, 13, 42). HBD-3 with 37% of sequence identity (Fig. 4B) presents a smaller r.m.s.d. at 0.94 Å for 28 aligned residues (Table III). Similarly, assuming that the data are comparable, Sphe-2 is as potent as HBD-3 on *E. coli* and *P. aeruginosa*, slightly more potent on *S. aureus*, and less efficient on *C. albicans*. In their modes of action, Sphe-2 and HBD-3 show similar levels of microbicidal activity against *S. aureus*, while against various Gram-negative strains Sphe-2 is less effective, having a bacteriostatic effect, and HBD-3 has a bactericidal effect (3, 43). Thus in terms of antimicrobial activity, Sphe-2 appears to be closer to HBD-3 than to HBD-2.

The sequence alignment of Sphe-2 and the three mammal β-defensins discussed above (Fig. 4B) does not reveal many conserved residues apart from the cysteine residues and two Gly-Xaa-Cys motifs. Concerning the distribution of residues at the surface of the molecule, the hydrophobic potential calculated at the Connolly surface with the MOLCAD option of SYBYL, using the same hydrophobic scale for Sphe-2, HBD-3, and HBD-2 (Fig. 3, central column), shows that HBD-3 is clearly the most hydrophilic molecule without any noticeable hydrophobic patch. Sphe-2 is hydrophilic but displays a hydrophobic patch including residues at the beginning of β2 and at the C terminus. HBD-2 and BNBD-12 exhibit an amphipathic structure (16, 19). Electrostatic potentials calculated at the Connolly surface with the MOLCAD option of SYBYL (Fig. 3, right column), using the same scale for the three molecules (+300, −300 kcal/mol) show that HBD-2, as expected from its total charge of +7, has the lowest positively charged surface. These charges are concentrated on the hydrophilic face of the molecule suggesting a surfactant-like mechanism of action.

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**Table III**

Scores of alignment of Sphe-2 with structurally related peptides

| Peptide | RK-1 | BNBD-12 | HBD-2 | HBD-3 |
|---------|------|---------|-------|-------|
| Percentage of identity | 28 | 37 | 18 | 37 |
| Structurally aligned residues | 10–14, 16–28, 29–37 | 10–27, 28–37 | 10–29, 30–37 | 10–28, 29–37 |
| Peptide | 3–7, 8–20, 23–31 | 10–27, 29–38 | 13–32, 34–41 | 16–34, 36–44 |
| r.m.s.d. (Å) | 1.31 | 1.33 | 1.25 | 0.94 |
| Number of aligned residues | 27 | 28 | 28 | 28 |
(16). Sphe-2 (10 positive charges, no negative charge) and HBD-3 (13 positive charges versus two negative charges) are both highly positively charged, and the charges are spread over their highly positively charged. The only exceptions concern the C termini of HBD-3, and there is no negatively charged residue favoring the formation of a Sphe-2 dimer very unlikely due to the strong electrostatic repulsion between the monomers. This suggests that the high efficacy of Sphe-2 against bacteria can be correlated with the presence of a hydrophobic patch, which seems common to avian defensins, and with its high cationic nature. In addition, we observed that the activity of Sphe-2 is preserved in salt conditions similar (judged through osmolality measurement) to the ones in the stomach content of penguin. This favors the hypothesis that Sphe-2, retaining its activity in vivo conditions, may regulate the bacterial flora in the penguin stomach, resulting in better food preservation. Finally Sphe-2 may represent a promising model for the structure-based drug design of salt-insensitive antimicrobial peptides to treat infections that result from the proliferation of bacteria and fungi in a salt-rich environment.

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