Initial Insights into Structure-Activity Relationships of Avian Defensins*

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**Background:** Avian defensins are antimicrobial peptides of a bird’s immunity.

**Results:** The target of chicken AvBD2 defensin is not chiral. Its structure is not amphipathic. The reduced and AvBD2-K31A forms dramatically decrease antibacterial activity.

**Conclusion:** AvBD2 may disrupt the bacterial membrane through a nonchiral, nonspecific interaction.

**Significance:** Knowledge of the structure-function relationships of avian defensins is a prerequisite for their use as alternatives to antibiotics.

Numerous β-defensins have been identified in birds, and the potential use of these peptides as alternatives to antibiotics has been proposed, in particular to fight antibiotic-resistant and zoonotic bacterial species. Little is known about the mechanism of antibacterial activity of avian β-defensins, and this study was carried out to obtain initial insights into the involvement of structural features or specific residues in the antimicrobial activity of chicken AvBD2. Chicken AvBD2 and its enantiomeric counterpart were chemically synthesized. Peptide elongation and oxidative folding were both optimized. The similar antimicrobial activity measured for both L- and D-proteins clearly indicates that there is no chiral partner. Therefore, the bacterial membrane is in all likelihood the primary target. Moreover, this work indicates that the three-dimensional fold is required for an optimal antimicrobial activity, in particular for Gram-positive bacterial strains. The three-dimensional NMR structure of chicken AvBD2 defensin displays the structural three-stranded antiparallel β-sheet characteristic of β-defensins. The surface of the molecule does not display any amphipathic character. In light of this new structure and of the king penguin AvBD103b defensin structure, the consensus sequence of the avian β-defensin family was analyzed. Well conserved residues were highlighted, and the potential strategic role of the lysine 31 residue of AvBD2 was emphasized. The synthetic AvBD2-K31A variant displayed substantial N-terminal structural modifications and a dramatic decrease in activity. Taken together, these results demonstrate the structural as well as the functional role of the critical lysine 31 residue in antimicrobial activity.

Defensins belong to a family of antimicrobial peptides characterized by cationicity, small size, β-sheet structure, and the presence of three disulfide bonds (1). Three subclasses (α, β, and θ) have been defined depending on the disulfide arrangement and the positioning of the six conserved cysteines. The α- and θ-defensin families have been considered to evolve by duplication and divergence from β-defensin ancestor genes because the former are not reported in evolutionarily old vertebrates such as fish and bird classes. Defensins play a major role in both innate and adaptive immunity (2). They have been found to be constitutively or inducibly expressed by neutrophils and epithelial cells from many mammals and birds, including chicken (1, 3, 4). They display a wide range of microbicidal or microbistic activities against Gram-negative and Gram-positive bacteria, fungi, and viruses (4). A substantial body of evidence indicates that the mechanism of action of defensins mainly relies on several structural features such as cationicity and amphipathy, which drive the antimicrobial peptide to interact with bacterial membranes and tend to divide peptides into two mechanistic classes, membrane-disruptive and non-membrane-disruptive (5, 6). In the latter case, there is growing evidence that defensins induce killing by acting on chiral anionic intracellular targets (for review see Refs. 7, 8).

Interest in defensins as therapeutic drugs is growing because defensins may constitute an alternative to the controversial use of antibiotics. In birds, a potential use of these peptides has been proposed in particular to fight antibiotic-resistant bacteria, including Salmonella, a major zoonotic agent that causes food poisoning (9). Numerous β-defensins were identified in birds from isolated peptides or gene sequences (for review see Ref. 4). In a previous study, it was shown that chicken β-defensin genes (avBD1 and -2) were highly expressed in the intestinal tissue of birds that are resistant to Salmonella colonization (10). Three defensins (AvBD1, AvBD2, and AvBD7) were therefore purified.
from chicken bone marrow, and their antimicrobial activity was tested on a series of Gram-positive and Gram-negative bacteria (11). Only chicken AvBD2 was shown to be more active against Gram-positive than Gram-negative strains, as reported for the king penguin spheniscin (AvBD103b) (12), the only other avian β-defensin whose three-dimensional structure has been determined to date.

Concerning the molecular patterns involved in the activity of avian defensins, the sole data currently available refer to ostrich AvBD1 and AvBD2 defensins, which share 39 and 78%, respectively, of identity with chicken AvBD2. Ostrich defensins were shown to create a slow and partial depolarization of the Escherichia coli membrane but were unable to provoke bacterium death by membrane disruption (13). This indicated that the ostrich defensins could cross the bacterial membrane to target a cytoplasmic molecule. Considering that the ostrich defensins were efficient in shifting the mobility of bacterial DNA in a gel electrophoresis assay, it has been proposed that DNA could be the target of defensin (13). In the context of the long term objective of improving knowledge of immunity in birds, this work was carried out to gain information on structure-activity relationships of the chicken AvBD2 defensin, at the atomic level, which is an essential first step to understanding how avian β-defensins function.

EXPERIMENTAL PROCEDURES

Reversed Phase High Performance Liquid Chromatography

HPLC analyses were carried out on either an Elite LaChrom system composed of a Hitachi L-2130 pump, a Hitachi L-2455 diode array detector, and a Hitachi L-2200 autosampler or a LaChrom 7000 system composed of a Merck-Hitachi L-7100 pump, a Merck-Hitachi L-7455 diode array detector, and a Merck-Hitachi D-7000 interface, which was also used for semi-preparative purification. The machines were equipped with C18 reversed phase columns (Nucleosil), 300 Å, 5 μm, 250 × 4.6 mm for the analytical separations, or 250 × 10.5 mm for purification. Solvents A and B containing 0.1% trifluoroacetic acid (TFA) were H₂O and MeCN, respectively.

Synthesis of the Linear S-Alkylated Defensins

Solid-phase peptide synthesis was run on automated synthesizer 433A from Applied Biosystems using Fmoc/t-butyl chemistry at a 0.1 mmol scale with O-(benzotriazol-1-yl)-N,N,N′,N′-tetramethyluronium hexafluorophosphate/1-hydroxybenzotriazole hydrate as the coupling reagent. Fmoc-Ala-methylphenoxyproionic acid (Polypeptide Group, France) (122 mg, 0.25 mmol) was manually coupled onto the aminomethylpolyethylene glycol polycrylamide resin (3 g wet weight, 0.1 mmol) in the presence of O-(7-aza-benzotriazol-1-yl)-N,N,N′,N′-tetramethyluronium hexafluorophosphate (95 mg, 0.25 mmol) and N,N-disopropylethylamine (86 μl, 0.5 mmol) for 2 h. The elongation was then carried out automatically using a 10-fold excess of protected amino acids and coupling reagents. The protecting groups used for the side chains were Arg(2,4,6,7-pentamethyl-dihydrobenzofuran-5-sulfonyl), Asn(triphenylmethyl), Cys(Acm),5 His(triphenylmethyl), Lys(t-butoxycarbonyl), Ser(t-butyl), Trp(t-butoxycarbonyl), Tyr(t-butyl). A 0.1-mmol scale program was used, and each coupling step was followed by capping with acetic anhydride. The coupling step was performed twice from Cys30 to Leu. The dipeptides Gly31–Ser and Gly31–Ser2 were also coupled twice, as the Fmoc-Gly-Ser(ΨMe,Me Pr)-OH pseudoprolin derivative (Merck). After completion of the peptide elongation, the peptide resin was treated for 3 h at room temperature with TFA/H₂O/i-Pr₂S/H₂O, 87.5:5:2.5:5, and the linear S-Acm-alkylated peptide was precipitated by dilution into ice-cold diethyl ether.

Synthesis of the Oxidized Defensins

In a syringe fitted with a frit, the S-Acm-protected peptide resin (15 μmol) was swelled in NMP (two times, 5 ml for 1 min). Silver tetrafluoroborate (58.4 mg per Acm group, 20 eq.) in NMP/H₂O, 9:1 mixture (4 ml), was transferred to the resin by suction, and the resulting suspension was stirred by rotation for 5 min at RT, in the absence of light, followed by washes with NMP/H₂O, 9:1, and then N,N-dimethylformamide. This treatment was repeated once (60 min of stirring), and the resin was further washed with pyridine (five times, 6 ml), then treated alternatively with sodium diethyldithiocarbamate (25 mM in NMP) and pyridine hydrochloride (1 M in CH₃Cl₂/MeOH, 95:5) (three and two times, 5 ml), followed by extensive washes with N,N-dimethylformamide. The peptide resin was then treated for 3 h at room temperature with TFA/H₂O/i-Pr₂S/H₂O/PhOH, 87.5:5:2.5:5, and the linear peptide was precipitated by dilution with ice-cold diethyl ether. The crude reduced form of AvBD2 was dissolved in 20% acetic acid (AcOH) and purified by semi-preparative C18 reversed phase HPLC.

The oxidative folding was performed at a peptide/GSH/GSSG molar ratio of 1:100:10 in deoxygenated MeCN, 200 mM Tris-HCl buffer, pH 8.5 (50:50, v/v), containing 1 mM EDTA. The peptide concentration (50 μg/ml) was measured using UV spectrophotometry at 280 nm (ε₉₀, 5579 M⁻¹ cm⁻¹). The kinetics of the oxidative folding was monitored by analytic C18 reversed phase HPLC. Aliquots (100 μl) of the folding reaction mixture were taken at regular intervals, and the reaction was stopped by adding 2 μl of TFA before HPLC analysis. The oxidative folding was quantitative over 30 min. The peptide was purified on a Resource S column (GE Healthcare) using a linear gradient of 0–0.5 M NaCl in 50 mM Tris, pH 7.5. The fractions corresponding to the pure peptide were loaded on a Sep-Pak® C18 column (6-ml column, Waters) followed by washing with 5% aqueous AcOH, eluted by MeCN/H₂O/ AcOH, 5:4:1, and lyophilized.

Mapping of Disulfide Bridges by Proteolytic Cleavage and Mass Spectrometry

Proteolytic Cleavage—Protein cleavages were performed in a total volume of 20 μl. To avoid the scrambling of disulfide bridges known to occur at basic pH, cleavages were performed in 30 mM ammonium acetate buffer adjusted to pH 6.5. Trypsin

5 The abbreviations used are: Acm, acetamidomethyl; MIC, minimal inhibitory concentration; Fmoc, N-(9-fluorenylmethoxy)carbonyl; NMP, N-methyl-2-pyrrolidone.
Structural Determinants for the AvBD2 Antibacterial Activity

Chemical Synthesis of AvBD2, Chemical and Functional Characterization Versus Extracted AvBD2

RESULTS

Antimicrobial Activity Test

The antibacterial activities of the peptides were measured by radial diffusion assay (16) as described in Derache et al. (11) in gel containing either one of the following Gram-positive bacterial strains: Bacillus cereus ATCC 14579, Staphylococcus aureus ATCC 29740, and Listeria monocytogenes strain EGD, or one of the following Gram-negative bacterial strains: E. coli ATCC 25922, Salmonella enterica serovar Enteritidis ATCC 13076, and S. enterica serovar Typhimurium ATCC 14028. For each bacterial strain, the statistical difference between native and variant peptide MICs was assessed by comparing the slope and intercepts of both regression lines with GraphPad Prism 5 software. The level of significance was set at p < 0.05.

Circular Dichroism Experiments

The CD experiments were carried out on a Jasco J-810 spectropolarimeter. Solutions of 30 μM (10 mM phosphate buffer, pH 7.2) of both L-AvBD2 and D-AvBD2 enantiomers were compared.

Three-dimensional NMR Structure

A standard set of two-dimensional 1H NMR experiments (COSY, 80-ms TOCSY and 160-ms NOESY) was performed on a 0.1 mM aqueous solution of the synthetic L-AvBD2 (H2O/D2O 90:10 and 100% D2O) at pH 4.1 and at 293 K. An additional set of data, recorded at 303 K, was used to resolve assignment ambiguities due to spin system overlaps. All spectra were recorded on a Bruker 800 MHz spectrometer (NMR Facilities, Gif-sur-Yvette, France). The NMR data sets were processed using the NMRPipe/NMRDraw software package (17, 18). 1H chemical shifts were assigned according to classical procedures (19). NOE cross-peaks were integrated and assigned within the NMRView software (17). Covalent bonds were built between the sulfur atoms of the paired cysteines. Structure calculations were performed with the ARIA 1.1 software (20). The calculations were initiated using the default parameters of ARIA and a first set of easily assigned NOEs. At the end of each run, the new assignments proposed by ARIA were checked manually and introduced (or not) in the following calculation. This iterative process was repeated until complete assignment of the NOESY map. A last run of 1000 structures was then performed with the final list of NOE-derived distance restraints, and 200 structures were submitted to the last step on ARIA. The 10 structures without residual NOE violation and with the lowest residual NOE energy were selected and considered as characteristic of the peptide structures. Representation and quantitative analysis of the calculated structures were performed using MOLMOL (21) and in-house programs.

The same sets of experiments were recorded on a VARIAN 600 MHz spectrometer for the variant AvBD2-K31A (2.4 mM of the synthetic peptide in aqueous solution at pH 4.2 and at 293 K). The same protocol was followed except that ambiguous constraints were introduced between cysteine residues, using the “ambiguous disulfide bridges” protocol of the ARIA 1.1 software (20).
group as a TFA-stable protection of cysteinyl residues to obtain the linear S-Acm-alkylated AvBD2 (AvBD2-Acm). To obtain the linear nonalkylated AvBD2 from the same batch of peptide resin, we developed conditions for the removal of the Acm groups on the peptide resin before the final TFA treatment. After HPLC purification of the reduced form of AvBD2, the oxidative folding was carried out using a procedure based on a thermodynamically controlled disulfide shuffling, in the presence of reduced and oxidized glutathione at pH 8.5. The folding kinetics was followed by quantitative analytical HPLC, and the reaction was shown to be complete in 30 min (supplemental Fig. S1). MALDI-TOF MS analysis of oxidized AvBD2 showed a 6-Da difference in mass compared with the reduced form, consistent with the fully oxidized form of this peptide (data not shown). The oxidized AvBD2 was then purified to homogeneity by cation exchange chromatography. Reversed phase HPLC analysis showed that synthetic AvBD2 co-eluted with the natural product extracted from chicken bone marrow (supplemental Fig. S2). As further evidence of the identity of the synthetic and natural peptides, their activities measured in MIC assays were in the same range for every bacterial strain tested (supplemental Table S1). Altogether, our data validated an efficient optimized protocol for the production of highly pure and biologically active synthetic AvBD2. It was successfully applied to the synthesis of the all-d-enantiomeric homologues of AvBD2 (d-AvBD2) and the AvBD2-K31A variant (supplemental Fig. S3). The all-d-form was checked by circular dichroism where CD spectra of the two enantiomers show equal and opposite spectra (supplemental Fig. S4). In the case of the AvBD2-K31A variant, the increase in hydrophobicity led to a poor folding yield. Organic solvents were then screened as folding additives (supplemental Table S2) and MeCN, which greatly enhanced the yield, was selected for preparative scale oxidative foldings. Our optimized protocol, including an efficient peptide elongation and the use of a co-solvent for the folding step, enabled an enhanced production yield up to 30–40% for all the AvBD2 peptides.

**Antibacterial Activity**

The antimicrobial activities of d-AvBD2 and l-AvBD2 were tested on a selection of three Gram-positive (B. cereus, L. monocytogenes, and S. aureus) and three Gram-negative (E. coli, S. enterica serovar Enteritidis, and S. enterica serovar Typhimurium) bacterial strains. As shown in Table 1, the MICs measured for the two enantiomers are identical for every tested strain.

To investigate the role of the well conserved three-dimensional frame of β-defensin in AvBD2 functionality, the antibacterial activities of the linear S-alkylated AvBD2 (AvBD2-Acm) were compared with the activities of its oxidatively folded counterpart (Table 1). The linear AvBD2-Acm is less active than the folded AvBD2 for every bacterial strain tested except for E. coli (p = 0.06), as shown by the dramatic increase in the MIC of AvBD2 when linear. In particular, the linear form of AvBD2 is 10 and 16 times less efficient than the folded peptide against the Gram-positive strains B. cereus (p = 0.0002) and L. monocytogenes (p = 0.0002), respectively. The linear form is even ineffective in our conditions toward the Gram-positive strain S. aureus, showing the strict requirement of the three-dimensional fold for an optimal antimicrobial activity. The effect of the three-dimensional structure on the activity is more limited for the Gram-negative strains. Indeed, for S. enterica serovar Enteritidis and S. enterica serovar Typhimurium, the linear form of AvBD2 displays an activity one and a half (p < 0.0001) to three times (p = 0.0002) lower than that of the folded AvBD2 peptide.

**AvBD2 Solution Structure**

**Partial Determination of AvBD2 Disulfide Bridge Arrays**—The determination of the correct disulfide pairing is generally achieved using enzymatic proteolysis of proteins and mass spectrometry analysis of the obtained cleavage products. These data can thus be introduced as additional constraints in structure calculations, allowing the three-dimensional models to converge more efficiently. For the chicken AvBD2 defensin, the
trypsin proteolysis experiment produced cleavage peptides that were identified by MALDI-TOF MS. The observed masses were matched to four sets of disulfide-connected peptides using the PeptideMap software (supplemental Table S3). Although peptides Leu1–Lys4 and Val20–Arg27 each contain one connectable cysteine, peptides Gly5–Lys19 and Ser28–Lys31 each have two cysteines that can participate in disulfide bridges. The connection of Leu1–Lys4 to Ser28–Lys31 shows that Cys3 is connected to Cys29 or Cys30. Similarly, Cys23 has to be linked to either Cys8 or Cys13. These first two connected products were observed with one linked cysteine and one free thiol. The products containing three connected peptides show that the remaining cysteine, either 8 or 13, forms a bond with the remaining cysteine 29 or 30. Trypsin cleavage thus narrowed down the number of possible disulfide bridges combinations to four (supplemental Fig. S5). Papain proteolysis produced one set of connected peptides, defining the Cys8–Cys23 bridge for certain. Consequently, in keeping with the trypsin result showing that Cys13 does not connect with Cys3, Cys13 can only connect with Cys29 or Cys30. However, these adjacent cysteines could not be differentiated. It is a known limitation of this method that, because of the impossibility to cleave between adjacent half-cystinyl residues, connected peptides containing a single disulfide bond cannot be obtained in such cases (22). Following analyses by enzymatic proteolysis combined with mass spectrometry, software computation, and logical deduction, among the 15 possibilities for the disulfide bridges array, only 2 remained: 3–29, 8–23, 13–30 or 3–30, 8–23, 13–29. Convergence to a well formed three-stranded \( \beta \)-sheet was only obtained in the first case, with a convenient residual number of NOE violations and satisfactory energies. The last iterations to refine the structure were then performed with the 3–29, 8–23, 13–30 disulfide bridges array. The final numbers of distance restraints used in the last run of ARIA calculations are detailed in Table 2.

![FIGURE 1. Alignment of the 32 avian defensin sequences currently referenced in Uniprot. Dots were inserted for alignment purpose. Left, protein name; right, Uniprot entry name and accession number; top, consensus sequence. Conserved residues are indicated in the consensus sequence; h indicates the conserved hydrophobic residues, + indicates the conserved positive residues.](image-url)
stabilized by a conserved array of three disulfide bridges. The sequential Cys29 and Cys30 belong to the middle strand of the β-sheet; therefore, their side chains point in opposite directions. The measured distances greater than 8 Å between the sulfur atoms of Cys13 and Cys29, or between Cys3 and Cys30, definitively preclude the possibility of C1–C6; C2–C4, and C3–C5 pairing that could match NOE NMR data. The structures were in very good agreement with the experimental data; there was no violation of distance restraints larger than 0.3 Å. Most of the residues (92.7%) were found in the most favorable regions of the Ramachandran plot. The whole, the secondary structure elements were well defined, and the root mean square deviation value calculated for secondary structures was 0.62 Å (Table 2). The analysis of the surface properties clearly indicated that the positive and hydrophobic residues were well distributed on the three-dimensional structure of the molecules (Fig. 2, B and C). Contrary to many antibacterial molecules, AvBD2 did not display any amphipathic character, neither along the primary structure (Fig. 1) nor on the three-dimensional structure of the molecules (Fig. 2).

Three-dimensional NMR Structure and Antibacterial Activity of AvBD2-K31 Variant

The protocol applied for AvBD2 was followed for AVBD2-K31A except that ambiguous constraints were introduced between cysteine residues, using the ambiguous disulfide bridges option. In the first calculations, each half-cystine was allowed to be linked to one of the five others, leading to 15 possibilities of pairing. During the calculations, each disulfide bridge is then allowed to float freely, and the protocol is driven to the most compatible disulfide bridges array, under the influence of the other NMR restraints. Once aberrant conformations (bridging more than two sulfur atoms) were discarded, a majority (72%) of structures correspond to the “3–29, 8–23, 13–30” disulfide bridges array, the residual 28% corresponding to the “3–30, 8–23, 13–29” pairing. At this stage, comparing two parallel calculations differing only by the disulfide bridges array imposed, 3–29, 8–23, 13–30 or 3–30, 8–23, 13–29, only the first calculations converged to a well formed three-stranded β-sheet. In the second calculations, the strands could not form properly. There was 25% more NOE violations, and the total energy was multiplied by 2. The refinement of the structure in the last iterations was then performed with the 3–29, 8–23, 13–30 disulfide bridges array.

A very accurate model of AvBD2-K31A variant was determined by NMR (the root mean square deviation value calculated for secondary structures is 0.19 Å; Table 2). The structures were in very good agreement with the experimental data, and most of the residues (96.2%) were found in the most favorable regions of the Ramachandran plot. On the whole, the secondary structure elements were well defined, and the root mean square deviation value calculated for secondary structures was 0.62 Å (Table 2). The analysis of the surface properties clearly indicated that the positive and hydrophobic residues were well distributed on the three-dimensional structure of the molecules (Fig. 2, B and C). Contrary to many antibacterial molecules, AvBD2 did not display any amphipathic character, neither along the primary structure (Fig. 1) nor on the three-dimensional structure of the molecules (Fig. 2).

### Table 2

**Structural statistics for the 10 final models of chicken AvBD2 and AvBD2-K31A variant**

|                  | AvBD2          | AvBD2-K31A     |
|------------------|----------------|----------------|
| NOE restraints   |                |                |
| Total            | 530            | 1069.1         |
| Intraresidue (i−j=0) | 278.7         | 295.7          |
| Sequential (i−j=1) | 112.3         | 261.1          |
| Medium range (2 ≤ | 29.2           | 170.5          |
| i−j ≤ 4)         |                |                |
| Long range (i−j=5) | 109.8         | 341.8          |
| Disulfide bridges|                |                |
| Introduced as constraints |        | Ambiguous disulfide bridges option |

| Root mean square deviation on backbone Ca atoms (pairwise, Å) |                |                |
|---------------------------------------------------------------|----------------|----------------|
| Global (2–35)                                                | 1.88 ± 0.48    | 0.37 ± 0.11    |
| Triple-stranded β-sheet                                     | 0.62 ± 0.13    | 0.19 ± 0.04    |
| β1                                                           | 0.32 ± 0.20    | 0.14 ± 0.05    |
| β2                                                           | 0.35 ± 0.12    | 0.11 ± 0.03    |
| β3                                                           | 0.28 ± 0.11    | 0.09 ± 0.03    |

| Ramachandran plot (%) |                |                |
|-----------------------|----------------|----------------|
| Most favored and additional allowed regions                  | 92.7           | 96.2           |
| Generously allowed regions                                    | 6.2            | 3.8            |
| Disallowed regions                                            | 1.1            | 0              |

| Energies (kcal mol⁻¹) |                |                |
|-----------------------|----------------|----------------|
| Electrostatic          | −1103 ± 90     | −1033 ± 66     |
| van der Waals          | −102 ± 19      | −76 ± 6        |
| Residual NOE energy    | 11 ± 5         | 36 ± 4         |
| Total energy           | −1024 ± 100    | −738 ± 46      |

* Data were determined by PROCHECK.
* Data were calculated with the standard parameters of ARIA.
peptides derived from its sequence were successfully synthesized in their linear or fully oxidized forms.

Involvement of a Chiral Partner in AvBD2 Antimicrobial Activity—To address the requirement of a chirality-dependent target in the antimicrobial activity, we synthesized and tested the all-D enantiomer of AvBD2. It is well established that the D-enantiomer of a native protein does not recognize the protein partners of the L-enantiomer or vice versa because of steric incompatibility (24, 25). In the field of antimicrobial peptides, it was shown earlier that the all-D-enantiomer homologues of magainins and cecropins exert antimicrobial potency comparable with the naturally occurring all-L-peptides, which indicates the absence of a specific receptor-mediated mechanism and the achiral lipid chains of the cell membrane as the main target (26–29). By contrast, during the discovery process of the outer membrane protein LptD as the chiral target of the peptidomimetic L27–11, it was shown that the all-D-enantiomer was essentially inactive (30). The measured MICs for both D- and L-AvBD2 enantiomers are identical toward various bacterial strains, either Gram-positives or Gram-negatives (Table 1). This clearly indicates that there is no chiral requirement for the antimicrobial activity. However, AvBD2 interacts with DNA in a gel shift assay (see supplemental Fig. S6) as reported for ostrich AvBD2 defensins (13), which share 78% of identity with chicken AvBD2 (Fig. 1). A similar behavior of AvBD2 and of its linear AvBD2-Acm form was observed in gel shift assays. These data suggested an unspecific interaction because of the cationic nature of the molecules, as already noticed for most antimicrobial peptides tested in vitro for their binding to nucleic acids (7).

Therefore, the bacterial membrane appears to be the AvBD2 target.

Importance of the Three-dimensional Structure in AvBD2 Antimicrobial Activity—Although the structural organization of most defensins stabilized by a network of disulfide bonds is crucial to maintain the antimicrobial activity, some linearized defensins retain their antimicrobial activity (31). On the series of bacterial strains used in our studies, the linear AvBD2-Acm peptide always proved to be less active than the fully oxidized form, indicating the requirement of the three-dimensional fold for optimal antimicrobial activity. This requirement is particu-
larly critical for Gram-positive strains. To draw the first structure-activity relationships for bird defensins, we determined the three-dimensional NMR structure of chicken AvBD2. It displays the structural characteristics of β-defensins, i.e. a three-stranded antiparallel β-sheet, stabilized by the conserved array of three disulfide bridges (C1-C5, C2-C4, and C3-C6). Most mammalian β-defensins display an additional N-terminal helix. The king penguin AvBD103b, the only avian defensin three-dimensional structure that is currently available, displays a high propensity of the N-terminal part to form a helix in aqueous solution (32). By contrast, chicken AvBD2 lacks the possibility to form an N-terminal α-helix because of its shorter sequence (Figs. 1 and 2A). Hence, this helix appears to be non-essential for antibacterial activity. Although other studies have shown that helix conformation is essential for the action on zwitterionic lipid membranes, this structural feature appears less significant for the permeabilization of negatively charged bilayers (33–35). This helix may be involved in activity against fungi or host-cell membrane and indeed involved in selectivity, as suggested by the fungicidal activity of AvBD103b (12) compared with the lack of AvBD2 potency against Candida albicans (36).

Structural Features—The ability of antimicrobial peptides to cross bacterial membranes and/or disrupt them is often governed by amphipathy (37–40). The analysis of the surface properties of chicken AvBD2 clearly showed that, contrary to many antibacterial molecules, AvBD2 did not display any amphipathic character (Fig. 2, B and C), even though it contains positively charged and hydrophobic residues. This organization of positive and hydrophobic residues, which are well distributed on the three-dimensional structure of the molecule (Fig. 2), certainly provides an appropriate equilibrium to interact with bacterial membranes.

To determine whether some of these positive and/or hydrophobic residues could play a role in this charge/hydrophobic equilibrium, the consensus sequence of the 32 avian β-defensins currently known was analyzed. As conserved residues often display a structural and/or a functional role in a given protein family, the consensus sequence was analyzed (Fig. 1) in the light of chicken AvBD2 and king penguin AvBD103b three-dimensional structures (32), which share 33% of sequence identity with AvBD2. Globally, the amino acid composition of avian β-defensins is highly variable, and only the six cysteines were strictly conserved (Fig. 1). These six cysteine residues, involved in a conserved array of three disulfide bridges, ensure the high stability of the molecule and the high resistance to enzymatic degradation and therefore undoubtedly have a structural role. For AvBD2, three half-cystines, one for each bridge, were totally embedded in the core of the protein. Their accessibility to the solvent calculated with NACCESS software (41) was 8.4, 0.0, and 0.2% for Cys8, Cys29, and Cys30, respectively. (For AvBD103b, the corresponding Cys5, Cys33, and Cys34 residues were totally embedded, with a solvent accessibility of 4.8, 0.1, and 0.3%, respectively). Subsequently, the consensus sequence highlighted two very well, but not strictly conserved, glycine residues (Gly37 and Gly38), belonging to Gly-Xaa-Cys motifs. Their role is most likely not only structural, but their presence could impact the neighboring residues. 1) Because of their small side chain, glycines are known to be highly flexible and to have a small steric size. At position 6, the short side chain of Gly6 prevented sterically clashes, i.e. in particular with the bulky well conserved Lys33 side chain of the “Cys-Cys-positive” motif (similarly, the totally embedded Gly10 of AvBD103b prevented steric clashes with the bulky well conserved Arg35). For two of the three exceptions not containing Gly at position 6 (mallard duck AvBD10 and chicken AvBD10, see Fig. 1), the Gly-Xaa-Cys and Cys-Cys-positive motifs are replaced by Gly-Xaa-Xaa-Cys and “Cys-Cys-Xaa-positive,” respectively (Fig. 1), which could ensure the same steric function. 2) The flexible and short side chain of Gly21 (Gly22 for AvBD103b), conserved in all 32 avian defensins except turkey AvBD3, chicken AvBD12, and chicken Gallins, was involved in a bulge where Val20–Gly21 in the second strand of the β-sheet are facing Cys29 in the third one (Ile2–Gly25 facing Cys33 for AvBD103b). This bulge could assist in placing the neighboring Val20 residue (or Ile24 in AvBD103b) in a favorable position, and/or it could ensure the proper folding of the protein (42), and/or it could give flexibility to this part of the protein (43). It is noticeable that this bulge is present in all the mammal β-defensin three-dimensional structures presently known: human hBD1–6, bovine BD12, and mouse mBD7–8 (Protein Data Bank codes 1kj5, 1fd3, 1kj6, 1zmm, 1zmp, 1zmq, 1bnb, 1e4t, and 1e4r, respectively). Moreover, the consensus sequence depicted in Fig. 1 highlighted well conserved positive residues at positions 4 and 31, and well conserved hydrophobic residues at positions 7, 10, 18, 20, and 26 (AvBD2 numbering), which did not seem to be involved in the fold itself, and consequently could have a functional role. The role of the well conserved hydrophobic residues at position 7 (but replaced by Ser in AvBD2) or at position 10 (but replaced by Arg in AvBD103b) is tricky to extrapolate with the only two three-dimensional structures available. They probably participate in the global hydrophobic/positive properties at the surface of the protein, as do the exposed Lys4 and Phe26 (Fig. 2D). Lysine 31 was pointed out (Arg35 in AvBD103b). This positive residue keeps only its charged extremity accessible to the solvent, whereas its hydrophobic side chain is surrounded by the hydrophobic N-terminal Leu1 and the well conserved Ile18 and Val20 residues, pointing toward the solvent (Fig. 2D). A similar feature is observed for AvBD103b, where the hydrophobic part of Arg35 lies in a hydrophobic environment provided by Ile22, Ile24, and Val37 pointing toward the solvent. In the case of AvBD103b, the positively charged extremity of Arg35, accessible to the solvent, was reinforced in the three-dimensional structure by two close additional positive charges, Arg8 and Arg9.

Role of Lys31 in the Antibacterial Activity and the Structure of AvBD2—To assess the structural role and to confirm, or reject, the functional role of the positively charged Lys31 in the mechanism of bacterial killing by AvBD2 and/or in its specificity toward different bacterial strains, the AvBD2-K31A variant was synthesized and studied. The point mutation of lysine 31 by an alanine residue (K31A) caused a dramatic decrease in activity (Table 1), showing the critical functional role of Lys31. However, this point mutation also causes a large structural modification in the N-terminal part of the molecule (Fig. 3), where an additional N-terminal β-strand is formed. A fine analysis of the
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three-dimensional models showed that the side chain interactions between the hydrophobic parts of Leu\(^3\) and Lys\(^{31}\), holding these residues in contact in AvBD2, are lost in the AvBD2-K31A variant. At this juncture it is not possible to precisely evaluate the contribution of these structural modifications to the decrease in activity. However, the critical functional and structural role of Lys\(^{31}\) has been evidenced.

Global Cationicity Versus Structural Distribution of Charges—A common feature of most antimicrobial peptides/proteins is their net positive charge, which is essential for the initial association with bacterial membranes, through electrostatic interactions with the anionic surface of bacteria. However, the specificity of each defensin is certainly linked to its own distribution of charged and hydrophobic residues on the one hand and to the differences in the membrane composition of bacteria cell membranes on the other hand (Gram-positive versus Gram-negative, or between species). From our results, the global cationicity of the molecule, which is reduced in the variant form of AvBD2-K31A, appears to be more critical for Gram-negative strains. This could be explained by the higher exposure of negative charges on the Gram-negative bacterial surface because of the lipopolysaccharide. Moreover, the discrepancy we have observed in this study between the Gram-positive and Gram-negative susceptibility to linear AvBD2 might thus come from their difference in bacterial membrane accessibility and composition (44, 45). However, the positive net charge of AvBD2 is one of the lowest among the avian β-defensins, and the AvBD2-K31A variant is charged only with three positive residues without losing all of its activity. In that variant, the loss of the three-dimensional structure has a dramatic effect on activity, as shown by the MIC of AvBD2-K31A-Acm, which was almost above the concentration range (Table 1). Thus, even if cationicity seems to be more important in the mechanism of action of AvBD2 against the Gram-negative bacteria than against the Gram-positive ones, the role of the three-dimensional structure, and the associated distribution of positive and hydrophobic residues at the surface, predominates in the activity of this avian β-defensin. In the absence of any amphipathic character, the interaction of AvBD2 with the bacterial membrane may be governed by an adequate distribution of positive and hydrophobic residues at the surface, which could be described as an appropriate partition constant (46). Recently, it has been proposed that synthetic α-helical amphiphilic antimicrobial peptides (47), and the amphiphilic human hBD3 (48), may act like “sand-in-a-gearbox.” This mechanism of action may be based on the ability of antimicrobial peptides to disrupt over space and/or time the highly dynamic membrane-bound protein complexes involved in essential processes of bacterial life. Even if not amphiphilic, AvBD2 could show an adequate partition constant to insert into the membrane, through nonchiral nonspecific interaction, and could disrupt the membrane equilibrium like sand in a gearbox.

Conclusion—The similar antimicrobial activity measured for both L- and D-enantiomeric chicken AvBD2 proteins clearly indicates that there is no chiral partner for the antimicrobial activity. Although the membrane emerges as the target, the resolution of the three-dimensional structure and the analysis of the AvBD2 surface revealed no amphiphilic distribution of its positively charged and hydrophobic residues. Thus, we propose that chicken AvBD2 antimicrobial activity may be based on a disorganization of the membrane through nonchiral nonspecific interaction.

Moreover, we highlighted a series of well conserved but not strictly conserved residues that could be involved in the antimicrobial properties and/or in the bacterial strain specificity of bird defensins. In particular, we pointed out lysine 31 of chicken AvBD2, lying in the hydrophobic environment provided by well conserved, accessible, hydrophobic residues. This study demonstrates the critical functional as well as structural role of Lys\(^{31}\) in antimicrobial activity.

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