Structural information on a cecropin-like synthetic peptide, Shiva-3 toxic to the sporogonic development of *Plasmodium berghei*

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This study is a contribution towards the understanding of the mode of action of Shiva-3 and more generally that of cecropin-like peptides. Structural information on Shiva-3 (a cecropin-like synthetic peptide) in water and in a membrane-mimicking environment (trifluoroethanol alcohol, SDS) were obtained using analytical centrifugation, CD and NMR spectroscopies. A total of 20 converged structures were retained on the basis of 197 non-redundant experimental constraints, including 166 distance constraints from the nuclear Overhauser effect measurements and 31 dihedral angle restraints derived from the purged COSY experiments. Some results obtained in presence of SDS are also presented.

The toxic effects of the peptides obtained by cleavage (trypsin and lysine-C hydrolysis) of Shiva-3 on *Escherichia coli* and on *Plasmodium berghei* sporogonic stages are reported. Biological effects are discussed in relation to the calculated structure. The antiparasite activity and the low mosquito toxicity of Shiva-3 make this peptide a good candidate for genetic transformation of mosquito vectors which warrants further studies aimed at the improvement of the molecule.

Keywords: 1H-NMR; cecropin-like; mixed solvents; plasmodium; shiva-3.

The evolutionary success of the insects is due to a variety of reasons, among which their potent antibacterial defense system [1, 2]. Their defense system includes a non-specific humoral reaction based on the secretion in the hemolymph of peptides with wide-range antibiotic activities. Cecropins, approximately 35–37 amino acids long [3], form part of this family of peptides [4–7]. They were initially discovered in the hemolymph of bacteria-challenged diapausing pupae of *Hyalophora cecropia* [4]. Cecropins were later isolated from a variety of insect species belonging to the orders Lepidoptera and Diptera. The main insect cecropins have a strongly basic N-terminal part linked to a neutral or less charged C-terminal part. The first three-dimensional structure to be solved was that of cecropin A [8, 9]. The peptide adopts a helix-bend-helix conformation with an amphipathic N-terminal helix and a more hydrophobic C-terminal helix.

The mode of action of cecropin is not fully understood but these peptides are thought to be selective for prokaryotic membranes due to their lipid composition [10–14]. Cecropins are most active against Gram-negative bacteria but they also have activity against some Gram-positive bacteria. Their primary mode of action appears to be that of membrane disruption followed by lysis. Their lytic effect on protozoa was initially tested in vitro using two synthetic peptides: SB-37, a closely related derivative of cecropin B, and Shiva-1, 60% different but with the same amphipathic and hydrophobic properties of the original molecules (Fig. 1). Both peptides induced a decrease in the growth and multiplication of *Plasmodium falciparum* and Trypanosoma cruzi, Shiva-1 being 10-fold more effective [15].

Transmission of malaria parasites by mosquito vectors is dependent on the successful sporogonic development of *Plasmodium* infective forms following ingestion of a blood meal infected with gametocytes [16]. The possibility of interrupting this development, either by selection and enhancement of refractory traits or by the introduction of parasiticidal compounds, has raised hopes for the production of genetically engineered mosquitoes to malaria-resistant parasites [17]. The lytic properties together with their simple structure have made cecropins potential candidates for this purpose.

The effect of Shiva-3, a peptide that differs from Shiva-1 by a single amino residue (tyrosine replacing tryptophan at position 4), on *in vitro* sporogonic stages of *P. berghei* in the midgut of *Anopheles albimanus* was investigated recently [18]. Peptide concentrations of 75 µM and 100 µM were effective in reducing ookinete production and the number of infected mosquitoes if applied in the first six hours of parasite development. The deleterious effect of the peptide on the parasite was effective after exposure for duration as short as 50 seconds. These observations raised the possibility of using Shiva-like peptide genes to engineer malaria-resistant vectors as an alternative in malaria control strategies.

Although the survival of Shiva-3 in mosquito midguts is about five minutes, degradation could be observed after the first
minute, therefore improvements in the peptide molecule are necessary to reduce its susceptibility to proteolysis and to increase its toxicity to the parasite. It is thus important to gain structural informations on Shiva-3 to evaluate the feasibility of introducing amino acid substitutions or stabilizing bonds.

In the present study, we have analysed the three-dimensional structure of Shiva-3 in water as well as in a membrane-mimicking environment using CD and NMR spectroscopies. We also report the effects of the peptides obtained by cleavage of Shiva-3 with trypsin and lysine-C endopeptidase on *P. berghei* and *Escherichia coli*. Biological effects are discussed in relation to the calculated structure. This study is a contribution towards the understanding of the mode of action of Shiva-3 and, more generally, that of cecropin-like peptides and should aim at proposing rational modifications of this molecule to improve efficiency.

### MATERIALS AND METHODS

**Materials.** Shiva-3 was synthesized in large quantities by solid-phase method using manual procedures already described [19], and purified on Sephadex G-10, followed by HPLC as reported [18]. The primary structure and the purity of the synthetic peptide were confirmed by HPLC. The amino acid composition of Shiva-3 was confirmed using a Beckman 6300 analyser, after acidic hydrolysis for 20 h at 110°C with 6 M HCl.

Deuterated compounds (d1-OH) 2,2,2-trifluoroethanol alcohol (99 atoms D/100 atoms), (d2-OH) 2,2,2-trifluoroethyl alcohol (99 atoms D/100 atoms), SDS (99 atoms D/100 atoms), sodium acetate (99 atoms D/100 atoms) and deuterium oxide (99.96% atoms) were purchased from Euriso-Top. Dodecyl β-D-maltoside was purchased from Calbiochem.

**CD experiments.** CD spectra were recorded at 20°C on a Jobin Yvon CD6 dichrograph with a 0.2-mm path-length cell. The ellipticity was calibrated with an aqueous solution of 0-camphorsulfonic acid [20]. Each spectrum was the result of averaging three successive scans at 90°C with 2 s integration/nm. The ellipticity values as a function of peptide concentration were measured from 5 µM to 50 µM and for the solvents H2O (pH 4–11), trifluoroethanol/H2O mixtures varying over 0–95% trifluoroethanol (by vol.) and detergent concentration (deoxy1 β-D-maltoside at 40 µM to 25 mM and SDS from 2 mM to 25 mM). The helical content was estimated using CD signal intensity according to previously published methods [21–23].

**Proton NMR experiments.** NMR spectra were recorded on a Varian Unity 500 spectrometer equipped with Sun Sparc 2 workstations. Data processing was carried out using the VNMR 5.1 program. Most spectra were recorded at 25°C on samples containing 0.7–1 mM peptide dissolved in [H2]trifluoroethanol/H2O 40% (by vol.) or in [H2]trifluoroethanol/D2O 40% (by vol.). The salt final concentration was 25 mM sodium acetate and the apparent pH was 5.0. The spectra were referred to the central component of the multiplet of the trifluoroethanol methylene resonance centered at 3.88 ppm, downfield from external tetramethylsilane.

Peptide spectra in SDS were recorded 10% D2O in H2O, pH 5.5, at 45°C. A 1.3 mM Shiva-3 in 200 mM detergent solution was prepared as previously published [24]. Spectra in SDS were referred to the water resonance at 4.58 ppm downfield from external tetramethylsilane.

The spectral width was 5000 Hz in H2O and 4200 Hz in D2O. Solvent proton resonance was reduced by low power irradiation during the recycling delay (1.5 s or 2 s), and during the mixing time for NOESY experiments. The residual transverse magnetization was reduced with a homospoil pulse applied at the beginning of both the relaxation delay and the mixing time.

Two-dimensional 1H-NMR experiments were collected in the phase-sensitive mode [25] with 2 K or 4 K (purged COSY experiments or micelles experiments) complex data points in t1, and 512 t1 increments with 32, 64 or 96 scans per t1 increment, depending on the experiment. Zero-filling was applied prior to Fourier transformation and data were processed with a squared sine bell for COSY and purged COSY which were shifted by 90° in the NOESY and TOCSY experiments.

Identification of spin systems was through analysis of spectra obtained in deuterated and protonated solvents using the following sequences: purged COSY for its sensitivity [26], double-quantum filtered COSY for its smaller intensity of diagonal peaks [27], total correlated spectroscopy (clean TOCSY) recorded with the MLEV-17 pulse scheme for the spin lock preceded by a 2-ms trim pulse and with 40, 60 and 80 ms isotropic mixing periods [28]. Through-space sequential assignments as well as NOE patterns were deduced from NOESY. To avoid zero-quantum coherence particularly embarrassing at short mixing times, a modified NOESY sequence was used (Sodano, P. and Delepierre, M., unpublished results), in which a 180° pulse was added during the mixing period with a varied position from scan to scan. NOEs were quantified using Felix software (Molecular Simulations) run on a Silicon Graphics Indy workstation. NOE build-up curves calculated for nine different peaks and for five mixing time values (50, 100, 150, 200 and 300 ms) showed that the spin diffusion effects were important only for mixing times greater than 200 ms.

Most NH-CoH scalar coupling constants were obtained from analysis of the NH-Hβ and NH-Hγ NOESY cross peaks using the program INFIT [29]. The CoH-CβH coupling constants were obtained directly from the purged COSY spectrum in D2O. The digital resolution values after zero filling along both co2s and co2s were 8.2 Hz and 1 Hz, respectively.

**Determination of dihedral and distance constraints.** 31 dihedral angle constraints were derived from the J(d,H)-d,Cα,Cα,d and NOE characteristics between prochiral protons. 22 β backbone torsion angles were restrained within a variable interval centered at –60°. For J(d,H)-d,Cα,Cα,d < 6 Hz, when associated with chemical shift index values smaller than –0.2 ppm [30] and with NOE characteristics of helicoidal structure, the β backbone torsion angles were constrained between –80° and –40°. If only two of the above elements were verified, the φ backbone torsion angles were constrained between –90° and –30°. The ranges of φ angle constraints were determined from a combined use of J(d,H)-d,Cα,Cα,d and NOE informations between Hα/Hβ and NH/ Hβ [31]. Using this procedure, the stereospecific assignments of some β protons were possible, and 9 γ angle conformations could be determined as g' . t and g- . Measured peak volumes from the NOESY experiment recorded with a 200-ns mixing time (the best compromise between restraints quality and restraints number) were converted into distance restraints. The calibration was based on the standard distance of 0.35 nm for the dα, 0.28 nm for dβ, and 0.34 nm for the dαβ, α-β cross-peaks in the α helix [32]. Pseudoatom corrections were used for degenerated protons such as methyl protons [33].

**Structure calculations.** Analysis of the obtained NOESY spectra yielded a set of 372 distance restraints. Among the 132
Peptide Inhibitory effect
no peptide added (control) 100 µM peptide %
1–2 0 47 ± 09
3 0 35 ± 10
4 0 45 ± 09
5 0 30 ± 23
6 0 19 ± 15
7 0 43 ± 22
8 0 24 ± 12
A 0 54 ± 08
B 0 41 ± 08
C 0 34 ± 14
Tri-Gly 0 06 ± 06
Shiva-3 0 100 ± 0

Table 1. Inhibitory effect of peptides from tryptic (residues 1–8) and lysine-C (A–C) digestion of Shiva-3 on the development of ookinetes from P. berghei. The inhibitory effect (± SD) was determined by counting ookinetes from samples containing 10000 red cells of the group treated against control experiments, for n = 5. Controls are cells cultured without addition of peptides, and numbers 1–8 correspond to the peptides purified after tryptic digestion, as shown in Fig. 2b. A–C are peptides digested with lysine-C and purified as indicated in the legend of Fig. 2a. Tri-Gly is a tripeptide of glycine, used as internal control for possible artefacts.

RESULTS

Effect of Shiva-3 fragments on E. coli and P. berghei sexual stage development in vivo. Aliquots of synthetic Shiva-3 were treated independently with trypsin and endopeptidase lysine-C and purified by HPLC. A C50 reverse-phase column was used for separation of peptides produced by enzymatic cleavage of Shiva-3, using a continuous gradient from solution A (0.12% trifluoroacetic acid in water) up to 60% solution B (0.005% trifluoroethanol/H2O mixture (40% by vol.), indicating the possibility of aggregation. No decrease in the linewidth as well as no change in chemical shifts were observed in spectra at a sample concentration as low as 40 µM, while the CD spectra obtained at different concentrations ranging over 5 µM–50 µM did not show significant changes of ellipticity. According to
Fig. 3. Far-ultraviolet CD spectra of Shiva-3 peptide in different solutions at 20°C. (A) In aqueous solution. Curves represent pH 4.11–10.7. (B) In trifluoroethanol/water solution. Curves represent 0, 10, 20, 25, 30, 40, 50, 60, 70, 80, 90 and 95% trifluoroethanol (by vol.). (C) The helix proportion is shown as function of trifluoroethanol concentration. (D) In SDS at concentrations ranging over 2–25 mM. (E) In dodecyl-β-D-maltoside at concentrations ranging from 40 µM to 25 mM.

Fig. 4. NH-HR region of TOCSY spectrum at pH = 5, 25°C, 40% trifluoroethanol (by vol.).

these results, either the peptide solution contains essentially monomers or the aggregates are extremely stable within the concentration range studied.

CD spectra and secondary structure. The CD spectrum of Shiva-3 in aqueous solution (Fig. 3A) showed no characteristics of secondary structure within a pH interval of 4–11. Addition of trifluoroethanol from 0% to 35% induced a large change in the CD spectra with a profile indicating the presence of α-helix structures (Fig. 3B). An isodichroic point was observed at 202 nm, suggesting a two-state transition, probably a helix-random coil interconversion. Addition of trifluoroethanol, from 35% to 80%, induced only minor changes in the CD spectra, while concentrations above 80% induced an increase of the helix content (Fig. 3C). Measurement of the ellipticity at 220 nm, [θ]220, at the plateau estimated the α-helical content as 40%.

Similar changes of the spectra shape were observed upon addition of SDS, an ionic detergent (critical micellar concentration, 4 mM; aggregation number, 66 [37]). At 5–25 mM SDS, the ellipticity remained constant at 208 nm and 220 nm, and the spectra obtained were characteristic of an α-helix (Fig. 3D). From the [θ]220, the α-helix content was estimated to be 42%, at
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2 mM SDS, and about 32% (±5%) at higher detergent concentrations. Contrastingly, spectra shapes similar to those obtained in H$_2$O were observed upon dodecyl-$\beta$-D-maltoside addition, a nonionic detergent (critical micellar concentration, 0.1 M) trifluoroethanol and H$_2$O at a 2:3 volume ratio for the structure observations combined with the results obtained by CD in the observed at residues 10–15 and 20–22 (data not shown). These observations combined with the results obtained by CD in the presence of trifluoroethanol drove us to choose a mixture of trifluoroethanol and H$_2$O at a 2:3 volume ratio for the structure determination by NMR.

The identification of the spin systems for each amino acid type [38–39] was achieved by means of through-bond connectivities (Fig. 4). Major difficulties in peak assignments came from extensive overlap of proton resonances, due to the large linewidth (about 10 Hz for amide protons) and to the weak dispersion of NH and Hα chemical shifts (430 Hz and 300 Hz). A set of two-dimensional experiments was performed in [${^1}$H$_2$]trifluoroethanol/D$_2$O in order to discriminate between exchangeable and non-exchangeable protons, such as the NH$_2$ side-chain protons of the seven Arg and those of the two Lys.

Sequential connectivities were obtained through the analysis of the short distances between the $\alpha$N, $\beta$N and NN protons of adjacent amino acids observed in two-dimensional NOESY spectra (Fig. 5). Knowing from the CD experiments that the molecule has a substantial helical content in the trifluoroethanol/H$_2$O mixture, the interactions between sequential residues were more likely than the interactions between residues far apart in the sequence. This assumption was used for the sequential assignment as well as for completing the spin-system identification, mainly from the $\alpha$$\beta$($i$,+$i$+3) interactions (Fig. 6; Table 2). The presence of an $\alpha$H-$\beta$H($i$,+$i$+1) NOE for the two Xaa-Pro dipeptide sequences suggests a high proportion of trans conformations.

The identification of secondary structures was performed through an analysis of patterns of NOE cross-peaks (Fig. 6). No long-range NOE indicative of a tertiary structure was identified. NN($i$,+$i$+1) connectivities of various intensities were observed all along the sequence between Arg3 and Gly38, except around the proline residues and for the Ile10–Asp11, Gln18–Gly20, Arg23–Gly25, Ile28–Leu30 and Ala34–Arg35 segments due to overlap with diagonal peaks. For most of the residues, the intensity of the $\alpha$N($i$,+$i$) NOEs cross-peak was greater than that of the $\alpha$N($i$,+$i$+1) cross-peak. The NN($i$,+$i$+1) cross peaks were more intense than the $\alpha$N($i$,+$i$+1) cross-peaks in the Arg5–Arg23 region and were associated with a considerable number of $\alpha$$\beta$($i$,+$i$+3) and $\alpha$N($i$,+$i$+3) NOE cross-peaks together with a few of NN($i$,+$i$+3) type. Contrastingly, in the C-terminal region, fragment 27–38, the NN($i$,+$i$+1) cross-peaks were often weaker than those of the N-terminal region and only two medium range NOEs ($i$,+$i$+3/$i$+4) were observed between residues Ile 28 and Val31/Gly32. Based on the NOE patterns obtained, that is (a) the intensities of NN($i$,+$i$+1) cross-peaks, (b) the low value for the $\alpha$N($i$,+$i$+1)/$\alpha$N($i$,+$i$) ratio and (c) the large number of medium range NOEs $\alpha$$\beta$($i$,+$i$+3) and $\alpha$N($i$,+$i$+3) at residues 6–24 and 27–32, $\alpha$-helical conformations are proposed for these two fragments. One $\alpha$N($i$,+$i$+2) and two NN($i$,+$i$+2) connectivities characteristic of 3$_{10}$-helix were also identified in the Asp11–Val13, Arg9–Asp11 and Ile17–Gln19 regions.

The presence of the two helices was confirmed by the small values of the $J_{\text{trans}}$ coupling constants ($<6$ Hz obtained for most residues in these regions except for Val13; Fig. 6). However, the linewidth of the amide proton is such that direct measurements of $J_{\text{trans}}$ coupling constants in the purged COSY experiment were overestimated and 19 coupling constant values were extracted from the NOESY spectrum using the INFIT program, while 11 small values ($J<6$ Hz) were measured directly. These values are in agreement with the secondary structures proposed above, with the exception of Val13 which has a $J_{\text{trans}}$ coupling constant greater than 6 Hz. It should be noted that in the C-terminal part, with the exception of Ala34 and Arg35, the J values near 7 Hz are compatible with a random-coil structure (Fig. 6).

The $\alpha$H proton chemical shifts are strongly dependent on the nature of the protein secondary structure [30, 40]; an uplift shift with respect to the random-coil value is observed for residues in $\alpha$ helices, while a downfield shift appears for residues in $\beta$ strands. Although these values are extracted from hexapeptide data obtained in water, the random coil H$_\alpha$ values are insensitive to the presence of trifluoroethanol up to 30% (±0.02 ppm) [41]. At higher trifluoroethanol concentrations, 40% [42] or 50% [43], the conformational shifts are in agreement with information on structural data obtained either from other NMR parameters or from CD measurements. The $\alpha$H secondary shifts for the majority of the Shiva-3 residues are

Fig. 5. NH-Hα and NH-NH regions of NOESY spectrum (200 ms mixing time), pH = 5, 25°C, 40% trifluoroethanol (by vol.),
indicative of $\alpha$-helix formation. On the basis of the magnitude of the $\Delta\delta$ conformational-dependent secondary shifts [44], and setting a threshold at 0.2 ppm, two helices were found extending over Arg3–Arg24 and at Ala27–Ala29. The smaller values of induced shifts, obtained for residues 14 and 20, were associated with the presence of glycine residues (Fig. 6).

Shiva-3 peptide can adopt an $\alpha$-helix conformation from residues 6–24 and 27–32. However, in the helical regions, the val-
Fig. 7. Superposition (residues 3–19) of the 20 structures of Shiva-3 calculated using DYANA. The figures were obtained with the program Molmol [56].

Fig. 8. NH-HR region of TOCSY spectrum at pH = 5, 45°C, 200 mM SDS.

ues of $J_{\text{HH}}$ coupling constants, although small ($<$ 6 Hz), are often greater than the expected theoretical value of 4 Hz [32], suggesting an equilibrium state between a helical and a more random structure. Thus, the relative stability of the helical fragments was probed through measurement of the exchange rates of amide protons with D$_2$O. At 25°C and pH 5, all amide protons were exchanged with deuterium within five minutes, emphasizing the weak stability of the Shiva-3 secondary structure.

Three-dimensional structure in trifluoroethanol/water mixtures. Despite the paucity of the NMR data, molecular modeling of the Shiva-3 structure together with backbone atoms superposition of the 20 best calculated structures is shown (Fig. 7). As expected from the number of available restraints, the calculated structures did not converge in the C-terminal part where no regular secondary structure could be defined. However, the N-terminal region represents a net helicoidal structure with a global rmsd value calculated for the backbone atoms between residues 3 and 19 of the 20 best calculated structures of 60 ± 24 pm. For these structures, the sum violation did not exceed 180 pm for distance restraints, 140 pm for Van der Waals’ restraints and 9.1° for angle restraints. A total of 92% of the residues in these structures appeared in the allowed regions of Ramachandran plot.

The structure contains a long helix spanning residues 5–19, with a kink around position 10 separating the first part of the structure, forming a $3_{\alpha}$ helix from the $\alpha$ helix extending over residues 10–16 and followed by a less defined helical structure that can extend up to residues 22/23 (as seen in eight of analyzed structures). Gly at position 20 appears to destabilizes the structure, leading to a smaller number of constraints in this region. An additional helix in the region at residues 27–32 appeared in seven of the best analysed structures. Several hydrogen bonds appeared persistently (at least 90%) in the calculated structures, corresponding to HN-O pairs of atoms at residues 6-3, 8-6, 13-9, 15-12, 16-12, 17-13, 18-15 and 19-16.
NMR experiments and secondary structure with 0.2 M SDS.
As in trifluoroethanol/H$_2$O mixture, the CD spectra in the SDS micelles revealed global characteristics of an 3-$\alpha$-helical secondary structure. The same methodology as described above was used for proton-resonance assignment, however the lack of information for scalar coupling from the TOCSY experiments led to incomplete identification of the spin systems (Fig. 8). Only spin systems corresponding to Val, Ala, Gly, the residues located in the C-terminal part, were easily identified. The spin system identification was completed through analysis of the NOESY experiments using the known assignments in trifluoroethanol/H$_2$O. Where possible, the sequence specific assignments were verified using the few unambiguous inter-residue cross-peaks in the two-dimensional NOESY. Finally, 75% of the backbone proton resonances were assigned unambiguously (Table 3). With the exception of Arg35, all of the Arg $\delta$H resonances were assigned through comparison with assignments obtained in trifluoroethanol. Only six of the seven arginine amide protons, the Hz of Pro2 and the whole spin system of Gly20 remained unassigned. Interestingly, spin systems not identified mainly corresponded to Arg residues, the positively charged residues. Assuming an electrostatic interaction with the micelles, the difficulties in identifying these residues could be caused by restricted motions [45]. Spin systems corresponding to the C-terminal part of Shiva-3 appeared more intense in the TOCSY experiment as a result of smaller linewidth (an average of 13 Hz for amide protons) as compared to the resonances in the N-terminal part (an average of 18 Hz for amide protons). Considering that the chemical shift index for the $\beta$H resonances indicates the presence of a helix from residues 5 to 22 [30], whereas the C-terminal part is in a random-coil conformation, the $J_{\text{min}}$ coupling constant should be smaller in the N-terminal region than in the C-terminal region. Consequently, the average linewidth for amide protons measured in the C-terminal part were overestimated. Qualitatively, the N-terminal part of Shiva-3 appears to be characterized by a larger $\tau$ than that of the C-terminal part, suggesting that the C-terminus is more flexible than the N-terminal.

**DISCUSSION**

Based on previous work carried out on cecropin and antibacterial peptides [8–10, 12, 14] the structure of Shiva-3 was probed in membrane-mimetic environments such as trifluoro-
ethanol, SDS and dodecyl β-D-maltoside. Water/alcohol mixtures are usually considered to approximate closely the dielectric constant of protein interiors and it has been suggested that a trifluoroethanol-enhanced α helicity is indicative of an overall α-helical propensity [46], leaving untouched those flexible parts of the peptide that have no preference for α helices. In fact, it would appear that addition of the trifluoroethanol to water stabilizes the helix only in the peptide regions forming a ‘nascent helix’ in water [47] that is, the helix structure within the context of existing helix/coil equilibrium [48]. There is a reasonable correlation between the trifluoroethanol-induced helicity and the helicity predicted on the amino acid sequence basis. Although the structure prediction for Shiva-3 peptide using the AGADIR program indicated α-helical structures nearly all along the chain with a higher tendency for the fragment 21–31 located in the C-terminal part of the molecule [49], no clear presence of secondary structure was found in aqueous buffer at 25°C. Only sparse NH-NH dipolar interactions at lower temperature, namely at 4°C, were observed, suggesting some structural organization of the peptide. Thus, the Shiva-3 conformation was determined in a mixture of trifluoroethanol and H2O.

The major problem faced when working in this solvent environment was the proton linewidth, which initially suggested an aggregation state of the molecule. The concentration effects on the CD spectra and on proton chemical shifts studies permitted the possibility of aggregation to be ruled out. It has been shown recently that, although many naturally occurring antibiotic peptides contain one or more features characteristic of coiled-coils, none of them met all the criteria required for the prediction of coiled-coil conformations [50], indicating that these peptides probably cannot aggregate as coiled-coils. The linewidth increase in the presence of trifluoroethanol might be simply related to the viscosity properties of the trifluoroethanol/water mixture itself. Indeed, the viscosity of the solvent increased by a factor of two in the presence of only 15% trifluoroethanol [51].

Although the Shiva-3 peptide forms α-helical structures in the trifluoroethanol/water mixture, it does not exist as a single conformation since the 1Hα average represents averaged values of conformational ensembles and, therefore, the structure presented here can be regarded as one of the highly populated conformational states in solution. From CD experiments, the α-helix content reached the same level in trifluoroethanol and SDS, that is 30–40%. Taking into account the relative stability of the helicoidal regions defined from the NMR experiments and based on the frequency of occurrence of these helices in the different structures generated, 37% α-helical structure is found.

On the basis of the NMR data and of the structure calculation described here, it could be interpreted that Shiva-3 contains two helices, one amphiphilic (helix I) and one more hydrophobic (helix II). Helix I extends over residues 5–24 with a more stable part in fragment 5–19, and helix II extends over residues 27–32. The importance of amphiphilic helices has been recognized in a number of antibacterial and lytic peptides. For cecropins and magainins, this helicoidal geometry is considered to be the key factor for the formation of the transmembrane pores which lead to cell lysis. For cecropin B and for Shiva-1, the predicted site for lethal action of the peptide is the cytoplasmic membrane [10, 52]. The mechanism proposed for cecropins involves the initial binding of the peptide through electrostatic attraction. Two helices were described for cecropin A, one encompassing residues 5–21 (helix I) and one encompassing residues 25–37 (helix II) [9]. It has been suggested that the primary anchor site for cecropin A on the membrane is provided by an aromatic residue (W or F) at position 2, which serves to bring the rest of the peptide into sufficiently close contact with the membrane for the helix formation to occur [14]. Then, the electrostatic interaction of the negatively charged membrane with the positively charged N-terminal stabilizes the conformation of the N-terminal helix (helix I). The main hydrophobic portion of helix II (residues 25–33) serves to further strengthen the interaction of cecropin A with the membrane thereby stabilizing the formation of helix I. It has been suggested that the major lytic activity of cecropin A resides in the amphiphilic helix I. Similar to cecropin A, the length of helix I of Shiva-3 is approximately 3 nm, that is similar to the thickness of the hydrophobic core of a phospholipid membrane, suggesting that helix I might penetrate into the bacterial membrane [53].

Once the peptide is structured, it can then rearrange itself in the membrane and two models have been proposed to explain the biological activity of cecropins. In the first model, developed from experimental data and physico-chemical principles, cecropin peptides form transmembrane pores [10–12]. In the second model, cecropin adopts an orientation parallel to the membrane surface and does not insert itself into the bilayer. A ‘carpet-like’ mechanism is proposed in which a layer of peptide monomers on the membrane surface destabilizes the phospholipid packing of the membrane, leading to its eventual disruption [14]. Solid-state 1H-NMR experiments of specifically labeled magainin in oriented bilayers have shown that the peptide adopts an α-helix structure that is immobilized via interactions with the phospholipids and is oriented parallel to the membrane surface [54].

The fact that in trifluoroethanol and SDS the C-terminal part of Shiva-3 is not stabilized is in favor of the ‘carpet-like’ model. Furthermore, the linewidth effect would suggest that the C-terminus exhibits faster motion in the presence of micelles than the N-terminal part, while the Arg side chains displayed reduced flexibility. Thus, the antimicrobial activity of the peptide might be correlated with its N-terminal region. This possibility is reinforced by the results obtained with the Shiva-3-cleaved peptides. The peptide corresponding to the N-terminal segment (residues 1–15) has the same effect as Shiva-3 itself. However, this is not exclusive to the N-terminal segment of Shiva-3, since the C-terminal segment also has a similar toxic effect. A plausible mechanism of action for Shiva-3 on the P. berghei development, according to the results reported here, is that these peptides might possibly form a ‘carpet-like’ complex with the biological membranes, disrupting their normal function, as originally suggested by Gazit et al. [14].

Another interesting observation (Table 1) is the lytic activity of short fragments generated by tryptic hydrolysis. The tripeptide Gin-Ile-Lys, produced by endopeptidase lysine-C, has a clear amphiphilic character, with the hydrophobic tail of Ile and the highly hydrophilic moity of the C-terminal Lys residue. This property could well simulate the effect of a possible detergent on the biological membranes. This was reinforced by the fact that another control tripeptide (made of Gly residues), which does not have hydrophobic and hydrophilic moieties is not toxic, even at high concentrations (100 μM). Most of the remaining peptides (Fig. 2) also have a highly hydrophilic region composed of charged residues (Lys and Arg) at the C-terminal part and a hydrophobic N-terminal amino acid, such as Val, Ala or Leu. It has been previously shown for antimicrobial peptides that, in addition to amphipathy, the juxtaposition of positive charges may play a significant role in the biological effect and that the formation of an α helix was not required [55]. This would be in agreement with the observation that the Shiva-3 positively charged residues have restricted motion in the presence of micelles. Thus, the mechanism of action of the fragments generated by enzymatic hydrolysis could well be non specific, because

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they somehow mimic detergent-like compounds. This possibility still remains to be experimentally demonstrated.

The structural information reported here provide insight for designing possible structural modifications of Shiva-3, in order to assess the features of the molecule, that could explain their lytic effects. At least three chemical modifications are planned for the production of rationally designed analogs of Shiva-3. The stability of the helix-I C-terminus can be enhanced by substituting Gln19 by Asp by homology with cecropin A for which the helix extends to residue 24 (Shiva-3 numbering). Similarly, the substitution of Gly20 for Pro would shorten helix I but also enhance its stability. Helix II, which was found to be very weakly stabilized despite a high degree of identity for the residues with cecropin A [9], could be stabilized by substituting Asp33 by Gln and probably also Arg35 by Thr. If the role of helix II is demonstrated, the last possibility would involve introduction of Cys residues at appropriate locations to maintain a fixed angle between helices I and II similar to the angle formed between cecropin A helices (70° to 100°). What would happen in terms of structure if these modifications are introduced and what would be its effect on its biological activity? Would the mutated analogs be more efficient for killing Plasmodium or more resistant to enzymatic hydrolysis? These questions can now be addressed and, certainly, will add to a better understanding of the lytic effect of Shiva-3.

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