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CONFORMATIONS OF GRAMICIDIN A AND ITS 9,11,13,15-PHENYLALANYL ANALOG IN DIMETHYL SULFOXIDE AND CHLOROFORM

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In order to understand the difference in single channel behavior of gramicidin A as compared to that of gramicidin M^- which is the mirror image of gramicidin M (all four tryptophanyl residues substituted by phenylalanine), conformational investigations were made under several experimental conditions. It is shown that, when examined under identical conditions, both molecules adopt the same conformations which could be identified in dimethyl sulfoxide (DMSO) and chloroform. In DMSO the conformation is based on a succession of β-turns while in chloroform gramicidin A and M^- can adopt a dimeric hybrid structure: a double helix terminated by two single-stranded helices involving the N- and C-terminal parts, respectively. It is therefore concluded that the difference in the energy profile between both gramicidins which was deduced from the ion transfer data has its origin in the nature of the aromatic side chains.

1. Introduction

Linear gramicidins:

HCO-L-Val-Gly-L-Ala-D-Leu-L-Ala-D-Val-
1 2 3 4 5 6
L-Val-D-Val-L-Trp-D-Leu-L-X-D-Leu-L-Trp-
7 8 9 10 11 12 13
D-Leu-L-Trp-NH-CH_2-CH_2-OH
14 15

(X-Trp, Phe, or Tyr for gramicidin A, B and C, respectively) are still attractive molecules as possible models of ion-conducting channels in biological membranes [1,2]. Therefore, numerous investigations, both theoretical and experimental, were undertaken in order to elucidate the conformation(s) of gramicidin A.

From the theoretical point of view it is shown that, for truly alternating poly-(DL)-peptides the α-helix is still a stable conformation [3,4], but also that, beside the DL ribbon structure [5] which is based on a succession of β-turns, other helices of new types can be built [4,6–9]. Contrary to the α-helix which is described on the basis of a single peptide unit, these new helices have dipeptides repeat units with torsion angles located in the β-region corresponding to the L and D residues, respectively. Two families of helices have been considered so far: (i) single-stranded π_{DL} helices; (ii) double-stranded $\pi\pi_{DL}$ helices with parallel or antiparallel strands. Both types of helices have a hole inside the helix which could act as a channel joining two aqueous phases separated by a lipid bilayer. The possible existence of the above-mentioned helices was confirmed by the study of model poly- and oligo-(DL)-peptides. Poly(γ-benzyl-DL-glutamate) was found by diffraction techniques to exist in various helical conformations, namely, the α_{DL} helix, the $\pi_{DL}^{4.4}$ helix [10] (the

lowest member of the family of π_{DL} helices) and four members of the double-helix family ($\pi\pi_{DL}^{5.6}$, $\pi\pi_{DL}^{7.2}$, $\pi\pi_{DL}^{9.0}$ and $\pi\pi_{DL}^{10.8}$ helices with antiparallel strands) depending on the history of the sample [11]. Later, the π_{DL}^{8} helix was proposed for two polypeptides which simulate the C-terminal half of the gramicidin A molecule; poly(D-Phe-L-Leu) and poly(D-Val-L-Val-D-Val-L-Ala) [12] and more recently it was shown by X-ray crystallography that the oligopeptides Boc-(L-Val-D-Val)$_n$OMe with $n = 4$ and 6 adopt a double-helical conformation [13–15]. It must also be mentioned that, although the strict alternation of D and L residues is required to build these helices, the nature of the side chains also plays a major role as pointed out in the study of poly(DL-alanine) which does not adopt a helical conformation but rather a sheet structure involving β-turns [16].

Concerning gramicidin A itself, in spite of numerous investigations including X-ray diffraction studies on crystals [17,18], its conformation is still disputed. While the crystallographic data are compatible with either a single-stranded π_{DL} helix or a double-stranded parallel or antiparallel $\pi\pi_{DL}$ helix, recently on the basis of ^{13}C-NMR experiments made on selectively labeled gramicidin A molecules, the double-helical form was ruled out, at least for the ion-gramicidin A complex in lipidic medium [19] and on the other hand the antiparallel $\pi\pi_{DL}^{5.6}$ helix was proposed for species 3 of gramicidin A when dissolved in dioxane [20]. Examination of the infrared spectra, particularly the amide I band, obtained on the various species [7,21] and on gramicidin A incorporated into dried vesicles [22] and comparison with those of oligo- or poly(DL)-peptides the conformations of which were unambiguously identified, also favour a β-like conformation and therefore a double-helical form. However, the origin of the shoulders observed from 1640 up to 1700 cm^{-1} is still in question. It was proposed that the origin could arise from a mixture of various conformations (single- and double-stranded helices) in equilibrium [21], but it was also suggested that the conformation of the polypeptide backbone could be heterogeneous [23].

The present paper deals with further conformational investigations on gramicidin A under various experimental conditions with the aid of a synthetic analog gramicidin M$^-$

$$\Big(\underset{1}{\text{HCO-D-Val}}\text{-}\underset{2}{\text{Gly}}\text{-D-}\underset{3}{\text{Ala}}\text{-L-}\underset{4}{\text{Leu}}\text{-D-}\underset{5}{\text{Ala}}\text{-L-}\underset{6}{\text{Val}}\text{-}$$

$$\text{D-}\underset{7}{\text{Val}}\text{-L-}\underset{8}{\text{Val}}\text{-D-}\underset{9}{\text{Phe}}\text{-L-}\underset{10}{\text{Leu}}\text{-D-}\underset{11}{\text{Phe}}\text{-L-}\underset{12}{\text{Leu}}\text{-}$$

$$\text{D-}\underset{13}{\text{Phe}}\text{-L-}\underset{14}{\text{Leu}}\text{-D-}\underset{15}{\text{Phe}}\text{-NH-CH}_2\text{-CH}_2\text{-OH}\Big)\ [24]$$

which was at the origin of this work. This analog was shown, in contrast to gramicidin A, to have a voltage-dependent single-channel behavior [25] and therefore it was of major importance to compare the conformation(s) of both the natural and the synthetic molecules in order to determine the origin of the difference in energy profiles between both molecules.

2. Materials and methods

Gramicidin A (a mixture of gramicidin A, B and C; 85, 10 and 5%, respectively) (Sigma) was recrystallized from ethanol before use. Gramicidin M$^-$ has the same origin as described previously [24]. DMSO d_6 and C^2HCl_3 (CEA) were used without further purification. ^{1}H-NMR spectra were recorded in 5-mm tubes using a Bruker WM 360 spectrometer working in the Fourier transform mode.

Assignments of the resonances were made using conventional decoupling techniques and two-dimensional correlation (COSY), relayed correlation (RELSY) [20] and nuclear Overhauser enhanced correlation (NOESY) spectroscopy.

CD spectra were obtained with a model III Jobin-Yvon dichrograph. Vesicles were prepared following the procedure of Weinstein et al. [26] using β,γ-dimyristoyl-L-α-phosphatidylcholine (DMPC) (Calbiochem). Viscosity measurements were made using a semi-micro Cannon Ubbelohde size 25 viscosimeter.

3. Results and discussion

3.1. Conformation in dimethyl sulfoxide

Owing to the greater amount of material, most of the results in dimethyl sulfoxide (DMSO) will

refer to gramicidin A. The infrared spectra of both gramicidin A and M^- when dissolved in DMSO are very similar (fig. 1), strongly suggesting that in this solvent both molecules have the same conformation. The spectra are characterized by broad amide I and II bands centered around 1665 and 1548 cm^{-1} respectively in accordance with that previously reported for gramicidin A [27]. As already pointed out, rather than proving the existence of a structure, from the infrared characteristics it is possible to reject conformations. Therefore, on the basis of the structures identified on model poly-(DL)-peptides the existence of π_{DL} or $\pi\pi_{DL}$ helical conformations can be ruled out for both gramicidins in DMSO. The identification of the conformation of gramicidin A in DMSO was carried out on the basis of ^{1}H-NMR spectroscopy using two-dimensional techniques. Owing to the strong overlapping of the different resonance lines especially in the high-field region, only the NH-αCH resonances and therefore the $^3J_{NH\text{-}\alpha CH}$ coupling constants will be discussed here. Fig. 2 shows the two-dimensional RELSY spectrum of gramicidin A (upper panel) together with the one-dimensional representation (lower panel).

Unambiguous assignment of all the NH and αCH resonances was achieved through Overhauser effect measurements (2D NOESY spectra and conventional NOE measurements) and are reported in fig. 3 and table 1. In the same table, all the $^3J_{NH\text{-}\alpha CH}$ coupling constants are also reported together with the temperature coefficients of the NH resonances. Examination of the various data of table 1 calls for several comments with regard to the possible conformations of gramicidin A. The double-standed helices are, a priori, ruled out as it has been shown that in DMSO the antibiotic is in a monomeric state [7]. Could it be in a single-stranded π_{DL} helical form? There is strong evidence indicating that this is not so.

(i) The coupling constants are inconsistent with any possible π_{DL} helices.

(ii) As it is largely in a monomeric state and owing to the antiparallel arrangement of successive NH groups, one expected to find identical temperature effects for the residues located in the last N- and C-terminal helical turns, the NH groups of which are not hydrogen bonded.

(iii) All residues of the same chirality should have identical NH-αCH coupling constants.

All these points are in opposition to the experimental findings. Finally, the α-helix is also ruled out as it requires, for a helix compound of both D and L residues, a set of two coupling constants (3.0 and 8.5 Hz) again in opposition with the experimental data. Taking into account the various values of the NH-αCH coupling constants which are compatible with β-turns we confirm, as already proposed, that the conformation of gramicidin A in DMSO is based on a succession of β-turns [28] (LD or DL bends). Examination of table 1, especially the values of the temperature coefficients, indicates a rather high solvent accessibility of the NH groups all along the molecule. This suggests that this conformation is in equilibrium between LD and DL bends with less flexibility in the C-terminal region as revealed by the alternation of highly and less accessible NH groups while all those located in the N-terminal part have nearly the same solvent accessibility. This is in accordance with the NMR relaxation time measurements [29,30] based on ^{13}C relaxation times. A substantially higher degree of mobility of the peptide backbone was found toward the N-terminal residue. The same conformation is also proposed for the synthetic analog as close coupling constants are observed (fig. 4, table 2). Note the

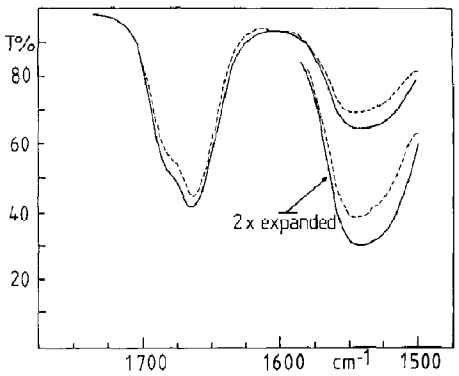

Fig. 1. Infrared spectra in DMSO at 25°C of gramicidin A (———) and gramicidin M^- (- - - - -). $C = 0.0154$ M in 0.05 mm thick CaF_2 cells. T, transmittance. The wave numbers are given in cm^{-1}.

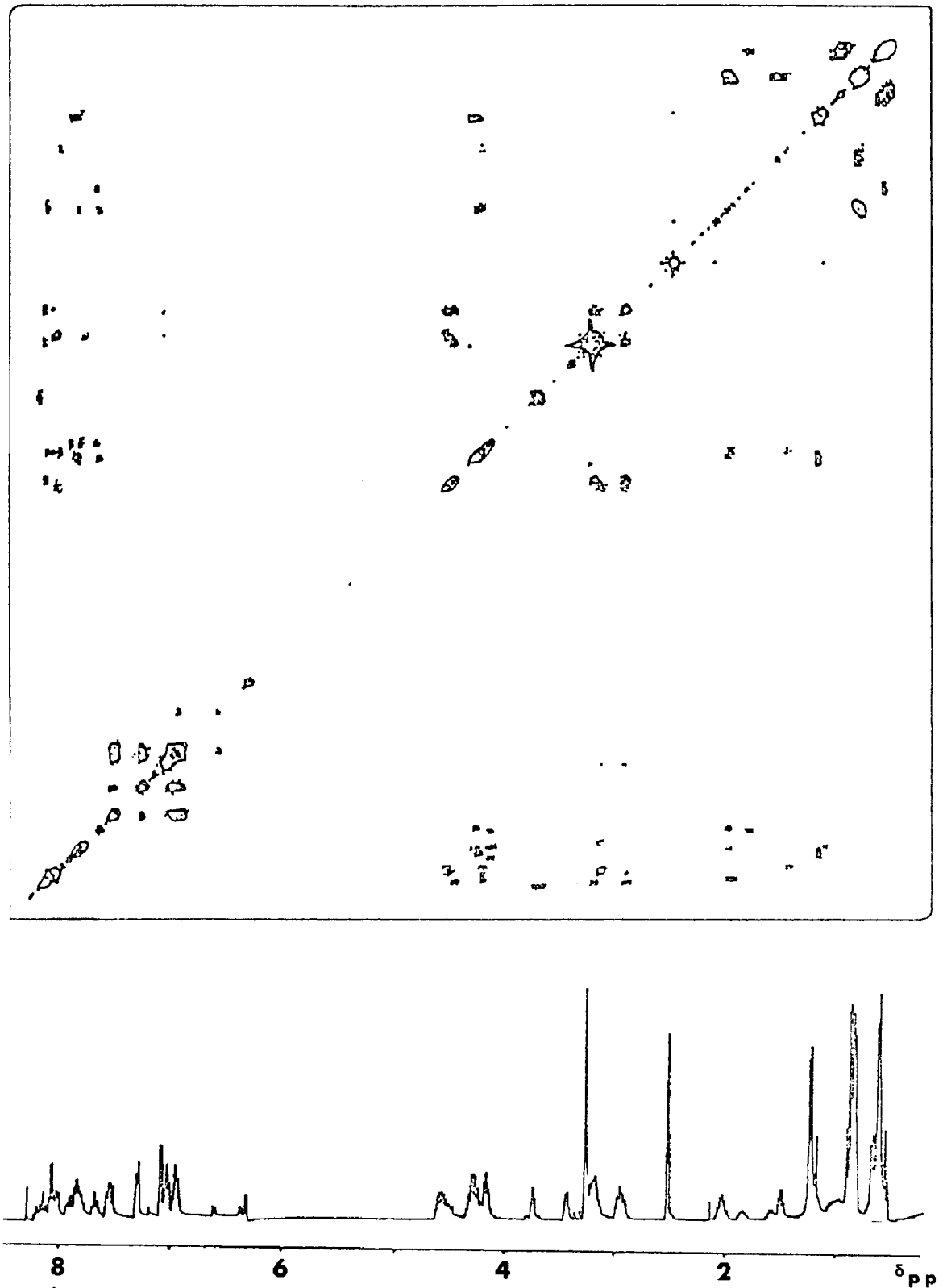

Fig. 2. ^{1}H-NMR spectra of gramicidin A in DMSO at 48°C. Upper panel, 2D RELSY spectrum; lower panel, 1D spectrum.

splitting of the low-field Phe NH resonance corroborating the higher rigidity of the C-terminal part of the molecule. The conformation proposed here for the two gramicidin molecules conflicts with that proposed previously by Glickson et al. [31] but is in full agreement with the results reported later by Heitz et al. [32] although they were less detailed owing to the lower field used for the NMR experiments.

3.2. *Conformations in chloroform*

Except for the spectra obtained in DMSO which are discussed above, all the known infrared spectra of gramicidin A are characterized by an amide I band centered in the 1633–1650 cm^{-1} region [7,21,22,24,27,33–37]. The situation in chloroform is of particular interest. Indeed, beside the major fact that it dissolves both gramicidin A and M^- (gramicidin A is sparingly soluble in pure chloroform, just enough for classical NMR measurements, but much more soluble in ethanol-stabilized chloroform which is suitable for other spectroscopic investigations), it allows infrared and NMR investigations. The infrared spectra of both gramicidins dissolved in chloroform at various concentrations are shown in fig. 5. This figure shows that upon dilution a transconformation occurs. The form obtained at higher concentration is characterized by an amide I band centered at 1634 cm^{-1} while that detectable at low concentration has an amide I band lying at 1648 cm^{-1}. This low-concentration form can be unambiguously attributed to the $\pi_{DL}^{4.4}$ single-stranded helix which

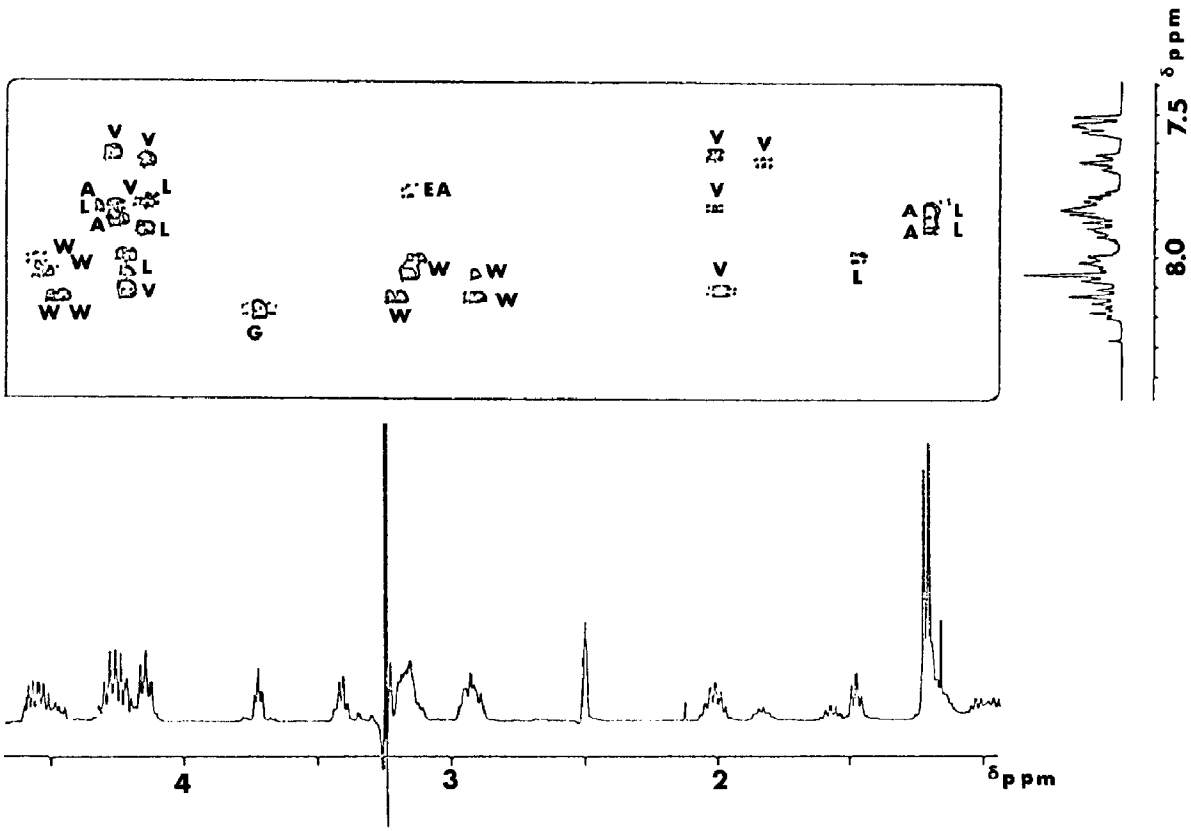

Fig. 3. Expansion of fig. 2 showing the assignments of the resonances (A, Ala; G, Gly; L, Leu; V, Val; W, Trp; EA, ethanolamine).

Table 1

Characteristics of the NH resonances of gramicidin A in DMSO

	Val_1	Gly_2	Ala_3	Leu_4	Ala_5	Val_6	Val_7	Val_8	Trp_9	Leu_{10}	Trp_{11}	Leu_{12}	Trp_{13}	Leu_{14}	Trp_{15}
$^3J_{NH\text{-}C_\alpha H}$	8.6 [a]	11.4 [b]	6.9	7.9	6.9	8.4	7.2	8.6	8.0	8.0	7.8	8.0	7.8	7.0	8.0
$\delta^{312\,K}$	8.12	8.19	7.89	8.02	7.84	7.72	7.84	7.75	8.01 or 7.81	7.83	8.07	7.81 or 7.83	8.07	7.92	8.15
$\frac{d\delta}{dt} \times 10^3$	4.0	4.0	3.4	4.0	3.9	3.0	3.1	3.4	4.3	3.9	5.2	3.9	5.2	4.3	6.0

[a] Quadruplet owing to the H-CO-NH coupling.

[b] Overall coupling constant.

was identified on poly(γ-benzyl-DL-glutamate) by diffraction data and characterized by an amide I band at 1648 cm^{-1} [15]. This low-concentration form can also be obtained through addition of hexafluoroisopropanol to concentrated solutions of gramicidins. Low- and high-concentration forms are doubtless monomer and dimer, respectively, as indicated by viscosity measurements (fig. 6) which show first a decrease of the viscosity (dimeric to monomeric form) and over 3% of hexafluoroisopropanol an increase of the viscosity attributed to a helix-random coil transition. Indeed, it is known that for low molecular weight polypeptides the helix-coil transition is accompanied by an increase of the viscosity [38]. This observation is consistent with previous findings which concluded a random coil structure for gramicidin A in hexafluoroisopropanol.

The constant governing the monomer-dimer equilibrium has been estimated from infrared measurements. Its value is approx. 3000 M^{-1} for gramicidin A and 30000 M^{-1} for the analog indicating a greater ability of the latter to form a dimer. Therefore, in the 1–2 mg/ml range used for the NMR measurements gramicidin M^- is mainly in a dimer form, while the nearly pure monomeric form in chloroform can only be obtained at very high dilution (fig. 5).

As to the conformation observed at the higher concentration, its identification was made on the basis of the NMR data. A first comment is suggested by the wide range of chemical shifts observed for the NH resonances (from 7.40 up to 9.20 ppm) indicating that we are dealing with a highly ordered structure. Note that two NH resonances are not seen on the spectrum as they are obscured by the aromatic resonances but can be detected on the two-dimensional spectrum (fig. 8).

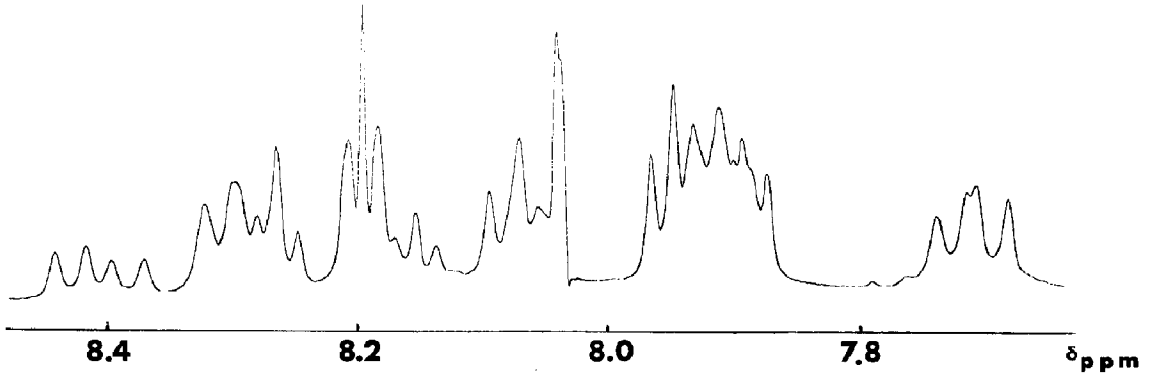

Fig. 4. ^{1}H-NMR spectrum of gramicidin M^- in DMSO (NH region).

Table 2

^{1}H-NMR data for gramicidin M$^-$ in DMSO

δ in ppm from TMS, J in Hz. The number of residues concerned is given in parentheses.

	NH		$C_\alpha H$	$C_\beta H$	$C_\gamma H_3$	
Val	δ	$J_{NH\text{-}C_\alpha H}^{obs}$	δ	δ	δ	
	7.70(1)	8.75	4.12(3)	1.78(1)	0.56(1)	
	7.73(1)	8.75	4.30(1)	1.98(3)	0.85(3)	
	8.19(1)	7.5				
	NH			$C_\alpha H_2$		
Gly	δ	$J_{NH\text{-}C_\alpha H_2}^{obs}$ (overall coupling constant)		δ		
	8.27	11.5		3.71		
	NH		$C_\alpha H$	$C_\beta H_3$		
Ala	δ	$J_{NH\text{-}C_\alpha H}^{obs}$	δ	δ		
	7.89(1)	6.30(1)	4.30(2)	1.19(2)		
	7.97(1)	6.50(1)				
	NH		$C_\alpha H$	$C_\beta H_2$	$C_\gamma' H$	$C_\delta H_3$
Leu	δ	$J_{NH\text{-}C_\alpha H}^{obs}$	δ	δ	δ	δ
	7.93(3)	7.5(1)	4.20(3)	1.13(3)	0.95(3)	0.67(3)
	8.09(1)	6.5(1)	4.30(1)	1.46(1)	1.55(1)	0.79(1)
	NH		$C_\alpha H$	$C_\beta H_2$	$C_\gamma H$	$C_\delta H_3$
Phe	δ	$J_{NH\text{-}C_\alpha H}^{obs}$	δ	δ	δ	δ
	8.20(1)	7.5				7.20(4)
	8.30(1)	7.5	4.45–4.60	2.82(4)		
	8.38(1)	9.0	(4)	3.00(4)		
	8.42(1)	8.75				

The chemical nature of all the NH resonance lines shown in fig. 7 was unambiguously assigned using conventional decoupling experiments and the corresponding $^3J_{NH\text{-}\alpha CH}$ coupling constants are reported in table 3. Examination of this table shows that, although they have the same chirality alanyl and phenylalanyl residues have different coupling constants. This strongly suggests that these residues adopt different conformational states and therefore are engaged in different conformational structures. Now which are these conformations? According to the Karplus equation: $^3J_{NH\text{-}\alpha CH} = A\cos^2\theta - B\cos\theta + C$ and using the coefficient given by Bystrov et al. [39] ($A = 9.4$, $B = 1.1$, $C = 0.4$) and an electronegativity correction $J_{cor} = 1.09J_{obs}$ the different possibilities for the N-C_α (ϕ) torsion angles are reported in table 3. Comparison of the possible ϕ angles with those expected for the various conformations which may accommodate both D and L residues suggest that the C-terminal part of gramicidin M$^-$, i.e., the D-Phe-L-Leu sequence, adopts a left-handed $\pi_{DL}^{4.4}$ helical conformation ($\phi_L = -130 \pm 10$, $\phi_D = 80 \pm 10$) while the remainder of the molecule adopts a double-helical conformation forming thus a hybrid structure similar to that proposed by Ivanov and Sychev [23]. Examination of molecular models (CPK) shows that such a hybrid helix can easily be built (fig. 9) when considering the central part of the dimer in an antiparallel $\pi\pi_{DL}^{5.6}$ double-helical conformation. However, the molecular models alone do not allow determination of the helical sense the same number of hydrogen bonds is found irrespective of the helical sense.

The left-handed helical sense which is deduced from the experimental data may be favored by different interactions arising from the side chains or by different hydrogen bonding induced by the ethanolamine moiety (OH---O=C for the left-handed helix and O^H---H–N for the right-

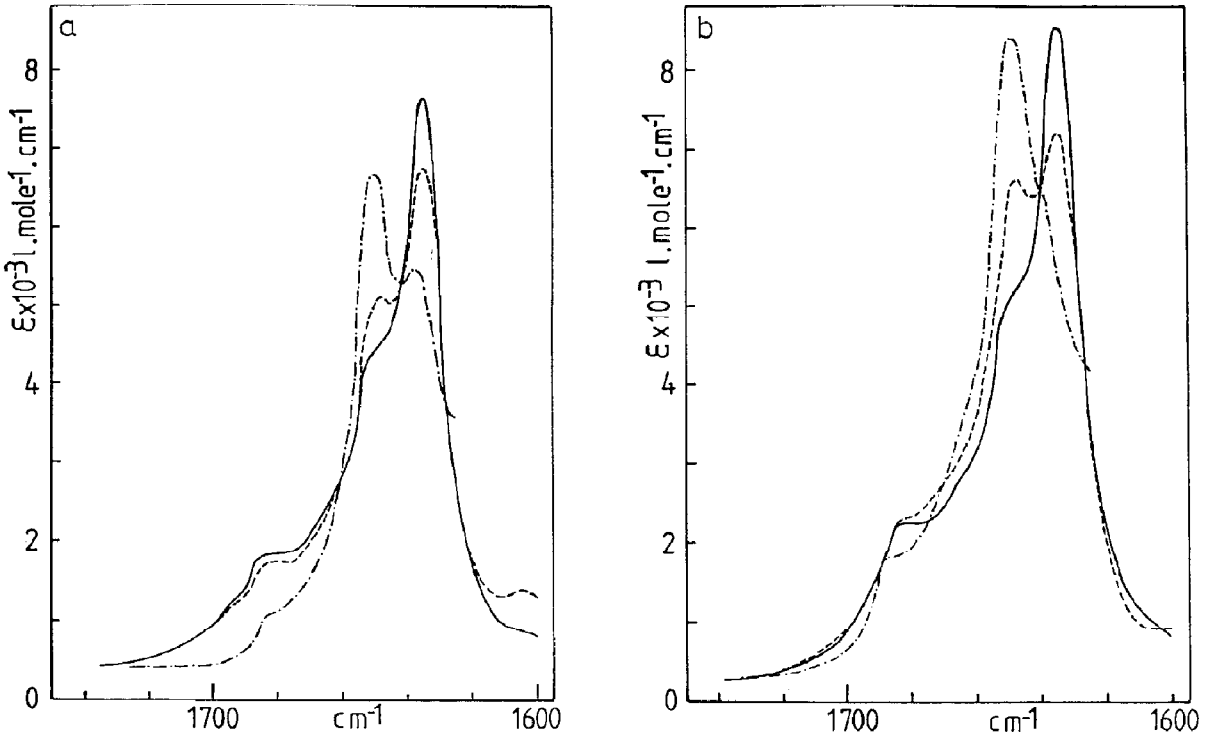

Fig. 5. Intrared absorbance spectra of gramicidin M$^-$ (a) and A (b) in chloroform at various concentrations: (———) 5 mg/ml, 0.1 mm thick cell; (- - - - -) 0.5 mg/ml, 1 mm thick cell; (·—·—·) 0.05 mg/ml, 1 mm thick cell expanded 10×.

handed one. Note that the reverse situation is also possible but less probable for steric reasons.

Further examination of the molecular models also shows that the length of this helical dimer is 31–32 Å, a value which should be compared with the length found for native crystals of gramicidin A (32 Å) as reported by Koeppe et al. [17]. Concerning gramicidin A, although the different assignments were made less accurately owing to the lower concentration used, the finding of identical coupling constants strongly suggests that in chloroform the natural molecule adopts the same hybrid conformation but, of course, with a right-handed helical sense. It is noteworthy that the ^{1}H-NMR spectrum, especially the NH region, could be solved only in the 1–2 mg/ml concentration range. At lower concentrations the signal/noise ratio was too small for allowing interpretation while an increase of the concentration leads to a broadening of all the resonance lines. This latter phenomenon is attributed to the formation of a pure double-helical structure very probably similar to that detected for gramicidin A in dioxane [20].

In addition, for gramicidin A, on the basis of ultraviolet and CD investigations which do not reveal any significant modification in the aromatic absorption region when varying the concentration, i.e.; by varying the monomer/dimer ratio, it can be concluded that the interactions involving the side chains are not modified. This is consistent with the finding of the hybrid structure proposed above as the tryptophan residues are located in an invariant structural region, namely the $\pi_{DL}^{4.4}$ single-stranded helix.

As to the screw sense proposed for gramicidin M$^-$ on the basis of the NMR data, it is consistent with the CD results shown in fig. 10, although

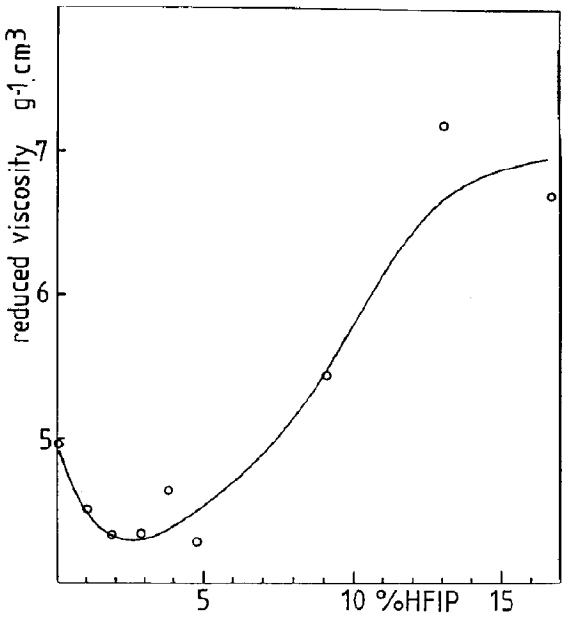

Fig. 6. Reduced viscosity at 25°C of a 1% solution of gramicidin A in chloroform as a function of the hexafluoroisopropanol (HFIP) content.

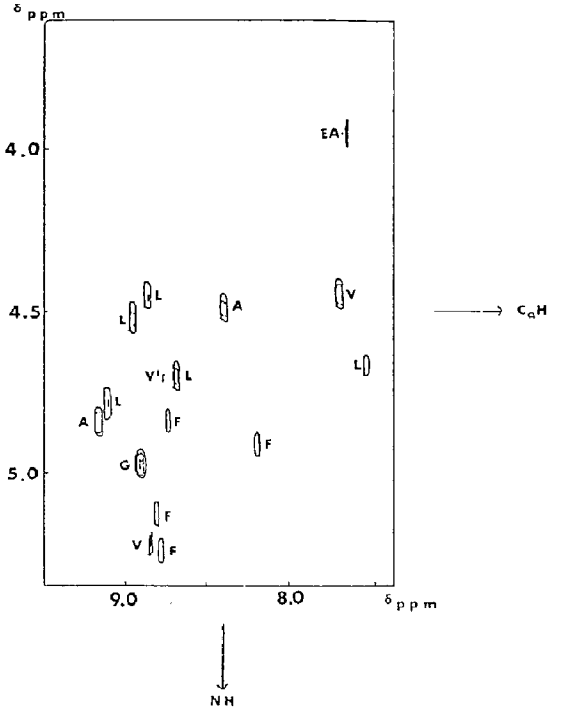

Fig. 8. 2D ^{1}H-NMR spectrum of gramicidin M$^-$ in chloroform.

they concern only the 230–260 nm region. While the sign of the ellipticity in the near ultraviolet region is mainly due to aromatic transitions for gramicidin A, this does not hold for gramicidin M$^-$. Indeed, as mentioned by Seipke et al. [40] who studied lysine-phenylalanine copolypeptides "All interpretations of the CD, spectra were made neglecting possible contributions of aromatic absorption bands. In the near UV region CD effects were not observed.... Hence, phenyl transitions lying in the peptide absorption region do not significantly contribute to the optical asymmetry." Therefore, a positive ellipticity as observed for gramicidin M$^-$ is thought to indicate a left-handed helical sense of the peptide backbone.

Finally, it should also to be pointed out that in all solvents used for CD investigations (methanol,

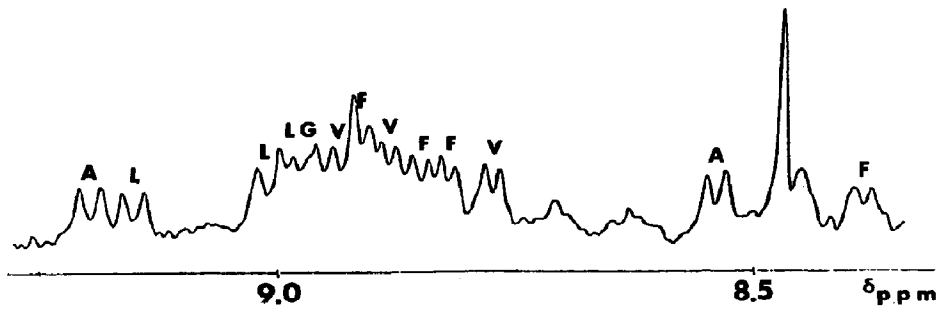

Fig. 7. ^{1}H-NMR spectrum of gramicidin M$^-$ in chloroform (NH region) (A, Ala; G, Gly; L, Leu; F, Phe; V, Val; EA, ethanolamine).

Table 3

Selected values of torsional angle ϕ determined from NMR $^3J_{\underline{N}H\text{-}\alpha\underline{C}H}$ coupling constants, compared to actual values

Residues	$J_{corr.}$ [a]	Selected values [b] (°) of		ϕ values [c] for left-handed	
		ϕ_D	ϕ_L	π_{DL}	$\pi\pi_{DL}^{5,6}$
D-Ala^{3,5}	9.26	144			121
	10.63	130			
D-Phe^{9,11,13,15}	6.76	80			
	6.81	80		83	
	7.36	83			
	7.63	85			
Leu^{10,12,14}	10.35		−134		
	10.63		−130	−131	
	10.90		−120		

[a] $J_{corr.} = 1.09 J_{obs.}$
[b] Determined from the relationship of Bystrov et al. [39].
[c] Reported in ref. 4.

n-butanol, dichloromethane, dioxane as well as incorporated into DMPC vesicles, the latter with or without ions) the spectrum remains nearly identical in the 230–260 nm region, indicating that under all these conditions gramicidin M$^-$ adopts the same screw sense. These observations seem to conflict with those of Wallace [41,42] who observed, for gramicidin A, a reversal of the CD spectrum. However, as already mentioned, owing to the strong overlapping of peptide and indole

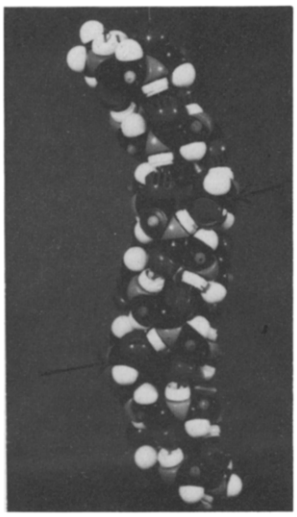

Fig. 9. CPK model of the structure of linear gramicidin in chloroform.

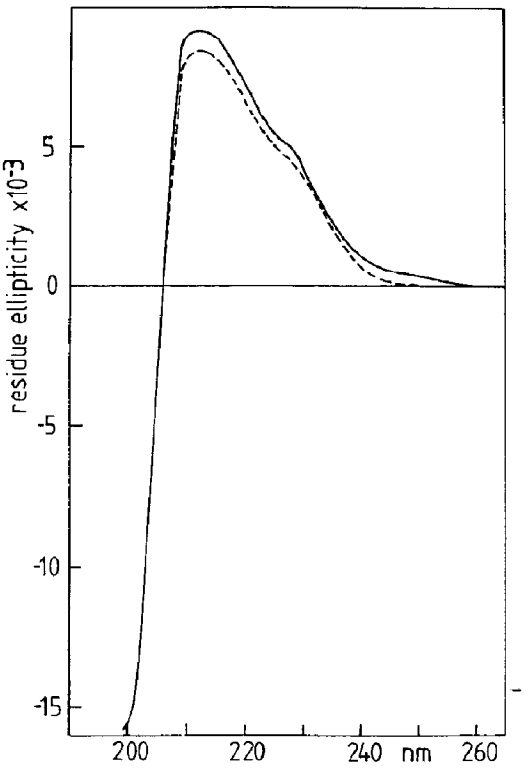

Fig. 10. CD spectra of gramicidin M$^-$ incorporated at 25 °C in DMPC (1:30) in pure water (———), 0.1 M CsCl (- - - - - -). $C = 1.18$ mg/ml, 0.1 mm thick cell.

absorption bands, a reversal of the sign of the optical activity may reflect a modification affecting only the side chains.

Conclusion

In conclusion of the present investigations it can be stated that, when observed under identical conditions, gramicidin A and M^- adopt the same conformations which were identified as a succession of β-turns in DMSO and as a hybrid of double- and single-stranded helices in chloroform. However, despite the identification of these conformations the question of the structure of the active form of the gramicidin channel is still open: double-stranded or head-to-head single-stranded dimer? Certain pieces of evidence would favour the head-to-head dimer as the single-channel experiments are performed at very high dilution conditions, but again the type of helix is not that identified in chloroform as the $\pi_{DL}^{4,4}$ helix cannot accommodate ions in its channel.

Nevertheless, the difference in the single channel behavior between both gramicidins [25] can be attributed to a side chain effect which modifies the energy profile of the channel. Now, we have to determine how can the side chains, which point outside the torus formed by the peptide backbone building the pore, influence the profile, regardless of the model: double- or single-stranded helix.

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