NMR solution conformation of gramicidin A double helix

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The conformation of species 3 of Val-gramicidin A in dioxane has been determined by two-dimensional NMR spectroscopy. It is represented by the left handed $\uparrow\pi\pi^{5.6}_{LD}$ double helix, a suitable model of an ion permeable pore across the membrane matrix.

Double helix *Ion channel* *Gramicidin A* *NMR* *Peptide conformation*

1. INTRODUCTION

The antibiotic gramicidin A (GA), an endogenous RNA polymerase inhibitor of the producing microorganism [1–3] has stimulated numerous studies for almost two decades, due to its ability to form well characterized ion permeable pores, or channels in the natural and artificial lipid bilayer membranes (see [4,5] and references therein). GA is a linear pentadecapeptide with strictly alternating LDLD... amino acid sequence (if glycine is considered as a potential D residue) and blocked end groups, formyl (Form) at the N-terminus and ethanol amide (EA) at the C-terminus. The transmembrane channel forming species is a dimer [6]. In organic solvents GA forms an equilibrated set of a large number of conformers, both monomeric and dimeric, depending on concentration, temperature and solvent composition [7,8]. In nonpolar media, for instance in dioxane, equilibrium is reached at such low rates that 4 dimeric species (so-called species 1–4) can be isolated in the individual state and in preparative amounts [7]. Comparative analysis of theoretically deduced and experimental IR spectra of various GA species and synthetic analogs complemented by CD data allowed the suggestion of a double helical conformation for all GA species [9]. However direct proof of these structures remains to be obtained.

In this study the spatial structure of the species 3 of GA in dioxane has been determined by two-dimensional NMR spectroscopy.

2. MATERIALS AND METHODS

Val-GA was obtained from gramicidin D (Serva) by countercurrent distribution [10]. The sample used for NMR studies contained 4–5% of Ile-GA as determined by amino acid analysis. The species 3 of GA was crystallized from ethanol at 20°C as in [7]. The crystals were dried under vacuum overnight to eliminate traces of ethanol.

For NMR studies were used freshly dissolved samples of 0.01 M GA in dioxane-d_8 (99% deuterium, Stohler Isotope Chemicals). The NMR spectra were recorded at 500 MHz on a Bruker WM 500 spectrometer. Chemical shifts are reported relative to tetramethylsilane as an internal standard. The COSY, SECSY and NOESY two-dimensional NMR spectra were recorded as in [11]. The two-dimensional relayed correlation spectroscopy (RELSY) was performed with the pulse sequence [12]:

$$90°-t_1-90°-\tau-180°-\tau-90°-t_2,$$

where t_1 and t_2 are the evolution and observation periods, respectively. The interval $90°-\tau-180°-\tau-90°$ induces two consecutive coherence transfer processes within the spin-coupled systems. RELSY

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spectra were obtained with $t_1 = 0.1–51.2$ ms (512 increments), $\tau = 32$ ms, $t_2 = 102.4$ ms. At the end of each pulse sequence the system was allowed to reach equilibrium during a fixed delay of 1.2 s (preparation period). To eliminate experimental artifacts and NOE cross peaks from the RELSY spectrum, groups of 32 pulse sequences with different phases were used for each t_1 value.

3. RESULTS AND DISCUSSION

During the 4 days required for recording a set of two-dimensional NMR spectra of GA in dioxane at 30°C no indication of conformational inhomogeneity (within the NMR time scale) has been observed (one signal per chemically equivalent proton). After 10 days at 30°C or 4 h at 90°C minor signals appear (5–7% of the spectrum intensity), presumably due to other conformational species of GA. Absorption of moisture from air causes changes in the chemical shift of some NH and $C^{\alpha}H$ signals. Special care was taken to avoid these processes.

Proton signal assignments to specific positions in the amino acid sequence (table 1) were carried out in two steps. From analysis of COSY and

Table 1

Chemical shifts δ (relative to internal tetramethylsilane) of the assigned ^1H NMR signals of the Val-gramicidin A species 3 in dioxane at 30°C, spin-spin coupling constants ^3J of the H-NC^{α}-H protons[a] and d_1- and d_3-connectivities of the amide proton of $i+1$ residue with, respectively, the C^{α} and C^{β} protons of the preceding i residue

Amino acid residue	NH	$C^{\alpha}H$	δ(±0.01 ppm) $C^{\beta}H$	Others	^3J (±0.3 Hz)	d_1	d_3
1	2	3	4	5	6	7	8
Form				HCO 9.47			
L-Val 1	9.16	5.65	3.27	$C^{\gamma}H_3$ 2.21, 2.21	9.2		
Gly 2	9.26	6.01, 4.70				yes	no
L-Ala 3	9.48	5.75	2.40		7.8	yes	
D-Leu 4	9.32	5.88	2.62, 2.52	$C^{\gamma}H$ 2.65 $C^{\delta}H_3$ 1.69, 1.91	8.0	yes	no
L-Ala 5	9.98	6.10	2.25		6.6	yes	
D-Val 6	10.44	6.25	3.41	$C^{\gamma}H_3$ 2.22, 2.22	9.2	yes	
L-Val 7	9.87	6.09	3.42	$C^{\gamma}H_3$ 2.15, 2.12	7.5	yes	
D-Val 8	10.58	6.14	3.40	$C^{\gamma}H_3$ 2.38, 2.38	9.2	yes	
L-Trp 9	10.44	6.68	4.49, 4.49	N_1H 10.31, C_2H 8.10 C_4H 8.89. C_5H 8.02 C_6H 8.17, C_7H 8.33	7.0	yes	
D-Leu 10	10.19	5.92	2.26, 2.13	$C^{\gamma}H$ 1.99 $C^{\delta}H_3$ 1.80, 1.51	9.3	yes	no
L-Trp 11	9.96	6.75	4.43, 4.30	N_1H 10.16, C_2H 8.20 C_4H 8.73, C_5H 8.09 C_6H 8.18, C_7H 8.30	7.4	yes	yes
D-Leu 12	10.65	5.96	2.17, 2.71	$C^{\gamma}H$ 2.56 $C^{\delta}H_3$ 2.00, 1.78	9.0	yes	no
L-Trp 13	10.25	6.36	4.48, 4.38	N_1H 10.75, C_2H 8.33 C_4H 8.98, C_5H 8.20 C_6H 8.28, C_7H 8.50	7.0	yes	yes
D-Leu 14	10.35	5.99	2.50, 2.40	$C^{\gamma}H$ 2.14 $C^{\delta}H_3$ 1.79, 1.74	9.2	yes	no
L-Trp 15	10.04	6.13	4.47, 4.15	N_1H 10.70, C_2H 8.23 C_4H 8.97, C_5H 8.07	7.2	yes	yes
EA	9.83	4.69, 4.23		C_6H 8.25, C_7H 8.45			

[a]The spin-spin coupling constants do not change their values on heating the sample from 30 to 90°C

SECSY spectra, spin systems of most amino acid residues were identified in the usual way [13,14]. In some cases COSY and SECSY spectra did not allow unambiguous assigments. For example, chemical shifts of C^{β} protons of 3 valine residues practically coincide (see table 1) and therefore spin systems of the NH-C^{α}H-C^{β}H protons of these residues cannot be unambiguously correlated with the $C^{\gamma}H_3$ protons. In this and other similar cases we used RELSY. For instance, in the case of the

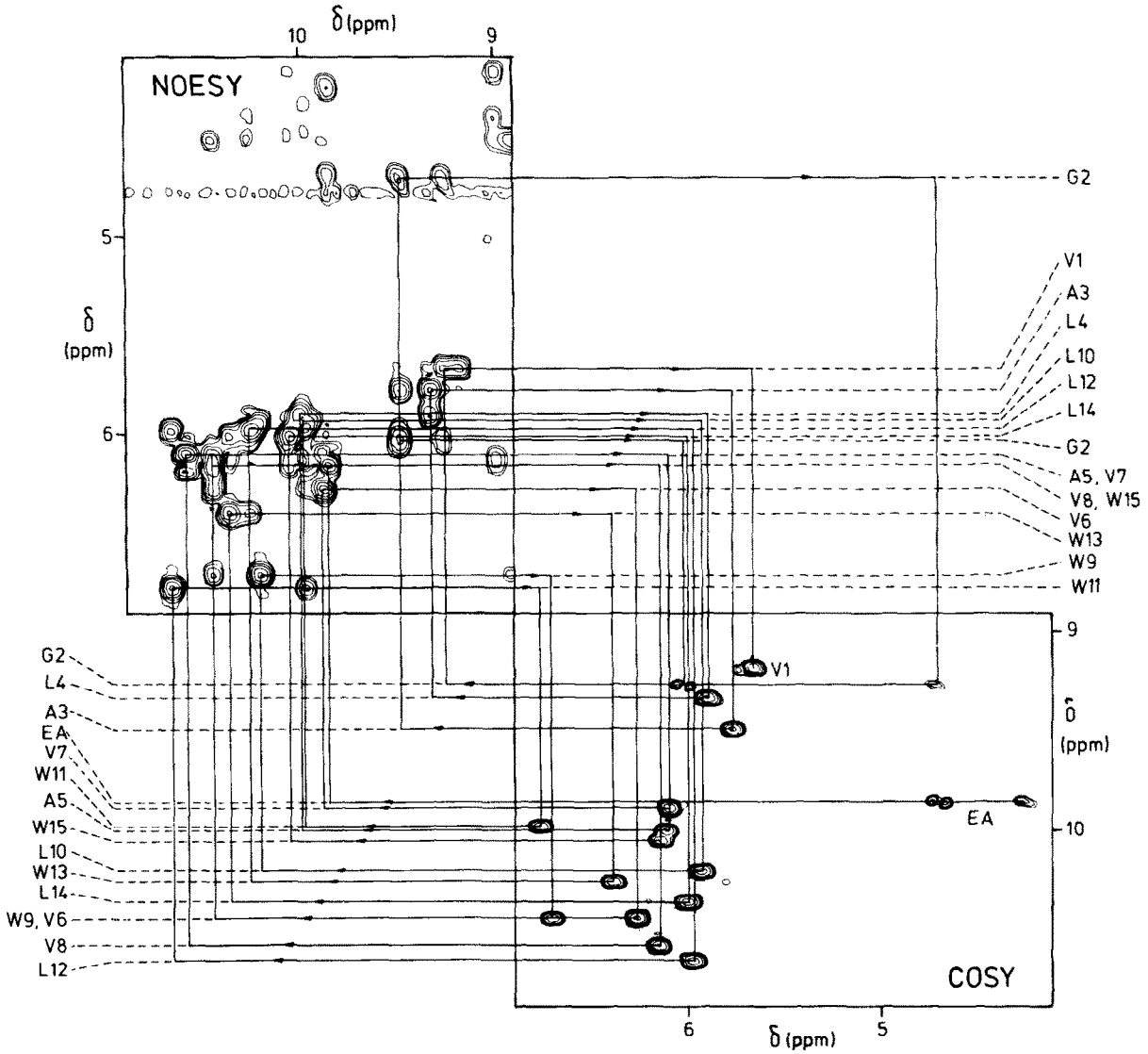

Fig. 1. Val-Gramicidin A species 3 combined COSY-NOESY connectivity diagram for sequential resonance asignments via NOE's between amide protons and C^{α} protons of the preceding residue (d_2). In the upper left, the region ($\omega_1 = 4.1$–6.9 ppm, $\omega_2 = 8.9$–10.9 ppm) of a ^{1}H NOESY spectrum of a 0.01 M solution in dioxane recorded at 30°C with mixing time 100 ms and digital resolution 4.9 Hz/point is presented. To the lower right, the region ($\omega_1 = 8.9$–10.9 ppm, $\omega_2 = 4.1$–6.9 ppm) of a ^{1}H COSY spectrum recorded from the same sample under identical conditions is shown. The straight lines and arrows indicate the connectivities between neighbouring residues from the C-terminal ethanol amide moiety towards the Val 1 residue. The amide proton chemical shifts are indicated by the assignments in the lower left-hand corner, and those of the C^{α}protons in the upper right-hand corner. The one letter code is used (Val, V; Gly, G; Ala, A; Leu, L; Trp, W).

valine residue the consecutive coherence transfer from C^{α} proton to C^{β} proton and then from C^{β} proton to $C^{\gamma}H_3$ protons gives rise to a cross peak in a RELSY spectrum with coordinates corresponding to chemical shifts of $C^{\alpha}H$ and $C^{\gamma}H_3$ protons. This allows unambiguous assignment of the HN-C^{α}H-C^{β}H$(C^{\gamma}H_3)_2$ spin systems of valine residues.

Spin systems of HN-C^{α}H-C^{β}H protons of tryptophan residues were linked to spin systems of indole protons through NOE connectivities between the C^{β} protons and the C4 and C2 protons, which are normally well observed in the NOESY spectra of polypeptides [15,16].

The spin systems of 2 Ala, 1 Gly, 4 Val, 4 Leu and 4 Trp residues, the N-terminal formyl and C-terminal ethanol amide groups were identified in the two-dimensional NMR spectra in accordance with the amino acid composition of GA.

Next, the spin systems were assigned to specific positions in the amino acid sequence by the conventional procedure of sequential resonance assignment through d_1, d_2 and d_3 connectivities for the amide proton of residue $i+1$ with, respectively, the $C^{\alpha}H$, the amide and the $C^{\beta}H$ protons of the preceding residue i [17]. Most of the assignments were based on d_1 connectivities (from the amide proton of the C-terminal ethanol amide moiety towards the C^{α} proton of the N-terminal valine, see fig.1). The d_2 connectivities were not observed, and only 4 d_3 connectivities were identified (table 1). It follows from the pattern of d connectivities [11,16], as well as from vicinal spin coupling constants of H-NC^{α}-H protons [18] (table 1), that the GA polypeptide backbone has an extended conformation. In addition, analysis of the NOESY spectrum (obtained under the conditions preventing spin diffusion, i.e. mixing time of 100 ms) revealed over 50 NOE connectivities between the residues which are non-adjacent to each other in the amino acid sequence. Some of them are presented in table 2. The majority of amide NH groups slowly exchanges with deuterium indicating their participation in hydrogen bonding ($t_{1/2} < 12$ h, 4% CD_3OD in dioxane, 30°C); the Val 1, Gly 2 and, presumably, Leu 4 exchange somewhat faster ($t_{1/2} \sim 1$ h).

The data obtained unequivocally prove that the GA species 3 in dioxane is formed by two extended, equivalent and therefore antiparallel polypeptide chains rolled into a double helix. Moreover, the connectivities presented in table 2 are consistent only with the left-handed $\Uparrow\pi\pi^{5.6}_{LD}$ helix shown in fig.2. The helix is 3.6 nm long (as estimated from the distance between the formyl oxygens). The dimer has a C_2 symmetry axis perpendicular to the helix axis. Table 2 presents the backbone protons of two different chains of GA dimer which are separated by less than 0.35 nm. The dimer has 26 intermolecular hydrogen bonds (fig.2). Carbonyls of the N-terminal formyls, Gly 2 and Trp 15 as well as the amide protons of Val 1, Gly 2 and Leu 4 do not participate in hydrogen bonding. Accordingly, the amide NH signals of Val 1, Gly 2, Ala 3 and Leu 4 are shifted downfield (by 0.06–0.15 ppm) when traces of methanol or water are added to the solution.

Thus two-dimensional NMR spectroscopy corroborates the formerly proposed $\Uparrow\pi\pi^{5.6}_{LD}$ structure of GA species 3 in dioxane [9] and refines its hydrogen bonding pattern. On the other hand the NMR spectra do not reveal any indication of the minor $\vec{\pi}_{LD}\overleftarrow{\pi}_{LD}$ dimeric forms postulated in [9]. The structural properties of GA in solution such as

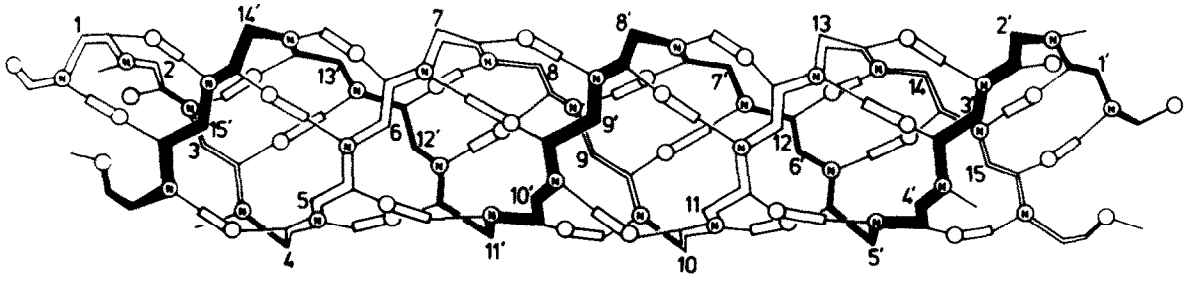

Fig. 2. Schematic presentation of the left-handed $\Uparrow\pi\pi^{5.6}_{LD}$ double helix of Val-gramicidin A species 3 in dioxane. Side chains are omitted for clarity. Intermolecular hydrogen bonds are shown by empty bars.

Table 2

Intermolecular contacts of backbone protons as predicted from a model of the Val-gramicidin A dimeric species 3 (fig. 2)[a] and observed by the NOE connectivities between the corresponding protons in NOESY spectrum (mixing time 100 ms)

NH-NH		$C^{\alpha}H$-$C^{\alpha}H$		NH-$C^{\alpha}H$	
Molecular model	Observation	Molecular model	Observation	Molecular model	Observation
V1-W15′	yes	V1-none	no	V1-none	no
G2-none	no	G2-L14′	[b]	G2-none	no
A3-W13′	yes	A3-none	no	A3-L14′	[c]
L4-EA′	yes	L4-L12′	[b]	L4-none	no
A5-W11′	[b]	A4-W15′	[b]	A4-L12′	yes
V6-L14′	[b]	V6-L10′	yes	V6-W15′	yes
V7-W9′	yes	V7-W13′	yes	V7-L10′	yes
V8-L12′	[b]	V8-V8′	[b]	V8-W13′	yes
W9-V7′	yes	W9-W11′	[b]	W9-V8′	yes
L10-L10′	[b]	L10-V6′	yes	L10-W11′	yes
W11-A5′	[b]	W11-W9′	[b]	W11-V6′	yes
L12-V8′	[b]	L12-L4′	[b]	L12-W9′	yes
W13-A3′	yes	W13-V7′	yes	W13-L4′	yes
L14-V6′	[b]	L14-G2′	[b]	L14-V7′	yes
W15-V1′	yes	W15-A5′	[b]	W15-G2′	[c]
EA-L4′	yes	EA-none	no	EA-A5′	[c]
				Form-EA′	yes

[a] The distances measured from the Dreiding model of the $\uparrow\downarrow\pi\pi^{5,6}_{LD}$ helix (fig. 2) between the NH-NH, $C^{\alpha}H$-$C^{\alpha}H$ and NH-$C^{\alpha}H$ protons are 0.34, 0.24 and 0.34 nm, respectively

[b] The NOE cross peak could not be revealed because its position is close to or coincides with the strong diagonal peak of a NOESY spectrum

[c] Existence of the NOE cross peak is uncertain because its position overlaps with another cross peak

~0.3 nm diameter of the axial cavity, hydrophobic external surface and ~3.6 nm length of the dimer seem to fit the requirements of the transmembrane channel. Analysis of the IR frequencies shows that there is no drastic difference between the structure of GA dimer in species 3 and in the dipalmitoylphosphatidylcholine liposomes [8,19,20]. Complete analysis of NMR data and the atomic coordinates of GA in dioxane will be published elsewhere.

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