NMR solution structure of gramicidin A complex with caesium cations

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The conformation of the 1 1 complex of [Val^1] gramicidin A with caesium cations has been determined in methanol/chloroform (1 1) solution by 2-dimensional ^{1}H-NMR spectroscopy The molecular structure was found to be a right-handed antiparallel double helical dimer $\downarrow\uparrow\pi\pi^{7\,2}_{LD}$ with 7 2 residues per turn, which incorporates two caesium cations

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

1. INTRODUCTION

Since it was reported [1,2] that gramicidin A (GA) forms transmembrane channels in biological and artificial membranes, this peptide has attracted much attention as a simple model of cation-selective pores (see [3,4] and references therein) It was shown that in black lipid membranes the channel is formed by two GA molecules [5] and accommodates simultaneously at least two monovalent cations in the inner cavity [6–8].

The spatial structure of the channel dimer was studied in model environments which pretend to mimic bilayer phospholipid membrane and/or which stabilize a cation–GA complex. Urry et al. [9] studied the thallium cation-induced ^{13}C NMR chemical shifts of carbonyl ^{13}C-labeled GA incorporated into lysolecithin micelles at relatively high peptide/lipid molar ratio (1:15). They interpreted the results obtained in favour of a transmembrane channel structure formed by a head-to-head dimer of left-handed, single-stranded $\overrightarrow{\pi}^{6\,3}_{LD}\overleftarrow{\pi}^{6\,3}_{LD}$ helices and rejected the double helical structures originally proposed by Veatch et al. [10]. The same conclusion was reached in the ^{13}C and ^{19}F NMR relaxation study of modified GA in phosphatidylcholine liposomes with use of paramagnetic probes [11]. However, it must be pointed out in regard to the above-mentioned cation-induced ^{13}C NMR signal shifts [9] that, as recently shown by energy calculations [12–14], the position of the cation in the inner cavity could deviate significantly from the channel axis of the GA dimer; this might complicate interpretation of the ^{13}C chemical shifts.

Cation–GA complexes are found to be stable in some organic solvents, whose properties can be considered similar to the interior of a bilayer membrane. The X-ray study [15] of crystals grown from methanol and ethanol solutions of GA complexes with Cs^+, K^+ and Tl^+ showed a molecule of cylindrical shape with one alkali cation per GA monomer, but was consistent with both single- and double-helical dimer conformations. The IR and laser Raman spectra of GA and Cs^+-GA crystals indicated a double helical structure for the dimers [16]. Conformational energy calculations [17,18] yield almost identical results for single- and double-stranded helices.

Some of the conformations of ion-free GA have been studied directly in organic solvent (dioxane) by NMR spectroscopy. The so-called species 3 and 4 [10] of GA were shown to be a left-handed antiparallel $\downarrow\uparrow\pi\pi^{5\,6}_{LD}$ and right-handed parallel $\uparrow\uparrow\pi\pi^{5\,6}_{LD}$ double helices, respectively [19–21].

Here, the spatial structure of a GA complex with caesium in a mixed methanol-chloroform solvent

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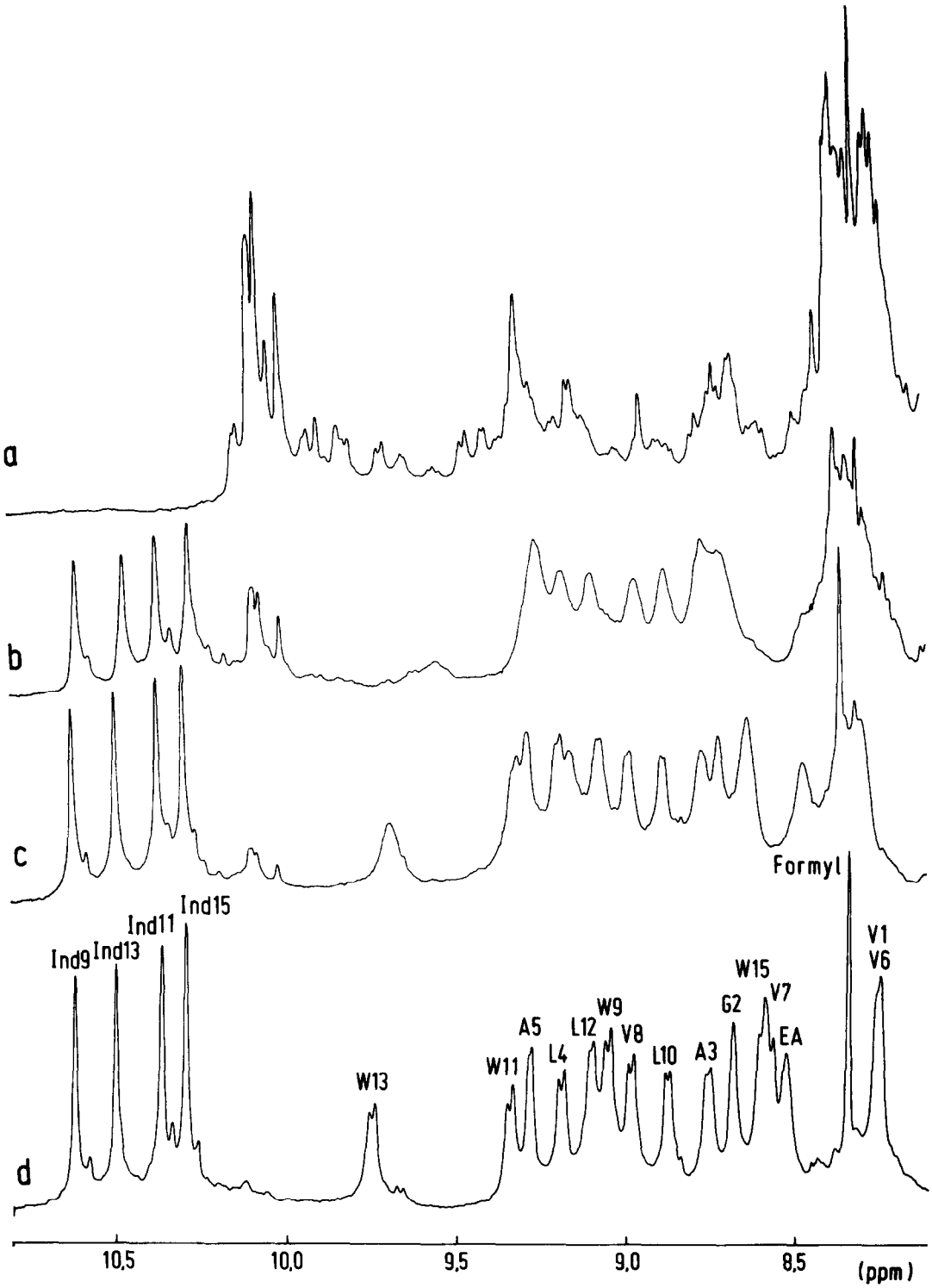

Fig 1 Low field region of the ^{1}H-NMR spectra of a 0 03 M solution of [Val^1]gramicidin A in $CD_3OH/CDCl_3$ (1 1), 30°C at different [Cs^+]/[GA] molar ratio· (a) 0 0, (b) 0 6, (c) 0.8 and (d) 1.1. The backbone NH resonance assignments are indicated by the one-letter code for the amino acid residues and the serial number in the primary structure of [Val^1]gramicidin A EA, amide proton of ethanolamine moiety; Ind, indole NH protons of tryptophan residues

has been determined by 2-dimensional (2D) NMR spectroscopy.

2. MATERIALS AND METHODS

GA was obtained from gramicidin D (Serva) by countercurrent distribution [22]. The sample used for NMR studies also contained 4–5% of [Ile^1]gramicidin A as determined by amino acid analysis. The solvents were methanol-d_4, methanol-d_3 (both 99% deuterium, Isotop USSR) and chloroform-d_1 (100% deuterium, Stohler Isotope Chemicals).

The proton 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 and NOESY 2D NMR spectra were obtained in a combined COSY-NOESY experiment [23]. Procedures and conditions for absolute mode of presentation of 2D NMR spectra are described in [24]. Fourier transformations to obtain the 2D NMR spectra in absorption mode of presentation were made by a modified version of the Bruker DISNMR program.

The IR spectrum was recorded on a Perkin-Elmer 180 spectrophotometer at ambient temperature

3. RESULTS AND DISCUSSION

The proton NMR spectrum of a salt-free solution of GA in $CD_3OH/CDCl_3$ (1 : 1) corresponds to a mixture of various forms of GA (fig.1a) presumably differing in conformation. When progressively increasing amounts of a 0.4 M solution of CsCNS in methanol-d_3 are added to the sample (simultaneously a corresponding volume of chloroform-d_1 is introduced to keep the 1 : 1 ratio of methanol/chloroform) a new set of signals appears and gradually grows in intensity (fig.1b–d). The chemical shifts of the new signals also change and their line widths narrow until the ratio $[Cs^+]/[GA]$ becomes ~1.0. The final spectrum corresponds to a single major conformational form of the Cs^+-Ga 1 : 1 complex with less than 15% admixture of minor components (fig.1d).

The behaviour of NMR spectra in the presence of caesium salt could be rationalized by the two-step equilibria [6]:

$$Cs^+ + GA_2 \underset{}{\overset{K_1}{\rightleftharpoons}} (Cs \cdot GA_2)^+ \quad (1)$$

$$Cs^+ + (Cs \cdot GA_2)^+ \overset{K_2}{\rightleftharpoons} (Cs_2 \cdot GA_2)^{2+} \quad (2)$$

in which the stable dimeric form of $(Cs_2 \cdot GA_2)^{2+}$ complex is demonstrated by NMR conformational analysis (see below). Both sites of cation binding are tight and identical for single occupancy. Presumably the binding of the first cation induces the GA dimer formation and its proper molecular structure. The $(Cs \cdot GA_2)^+$ complex is stable; its lifetime exceeds 30 ms, as indicated by the line width of NMR signals, and the equilibrium binding constant $K_1 \approx 10^4\ M^{-1}$. The second equilibrium ($K_2 \approx 3 \times 10^3\ M^{-1}$) is faster – lifetime of $(Cs_2 \cdot GA_2)^{2+}$ is <1 ms and therefore only the averaged NMR signals of $(Cs \cdot GA_2)^+$ and $(Cs_2 \cdot GA_2)^{2+}$ are observed. It can be assumed that when the second Cs^+ is accommodated, then both the identical sites become less tight due to electrostatic repulsion of the cations.

The conformation of a $Cs_2\ GA_2^{2+}$ complex was studied by 2D ^{1}H-NMR spectroscopy at $[Cs^+]/[GA] = 1.1$. First, the chemical shifts of most protons were assigned to specific positions in the amino acid sequence of GA (table 1) by the conventional procedure of sequential resonance assignments [25]: the proton spin systems of amino acid residues were identified in COSY spectra via J-connectivities and then they were aligned, via nuclear Overhauser effect (NOE) d-connectivities between neighbouring residues, into the amino acid sequence of GA. All the assignments from the C-terminal ethanolamine moiety towards the N-terminal valine residue are based on NOE d_1-connectivities between the amide NH proton of residue $i+1$ and the C^α proton of the preceding residue i in the sequence. The d_2-connectivities $N_{i+1}H \ldots N_iH$ were not observed and only a few d_3-connectivities $N_{i+1}H \ldots C_i^\beta H$ were identified in NOESY spectra (table 1).

Analysis of the NOESY spectra of the Cs^+–GA complex in $CD_3OD/CDCl_3$ (with amide protons exchanged for deuterium) and in $CD_3OH/CDCl_3$ solutions also reveals over 50 NOE connectivities between protons of non-adjacent residues in the amino acid sequence For example, fig.2 shows a fragment of the absorption mode NOESY spectrum with NOE cross-peaks between NH and $C^\alpha H$ protons.

Table 1

Chemical shifts δ (relative to internal tetramethylsilane) of the assigned ^{1}H-NMR signals of the [Val^1]gramicidin A complex with caesium in methanol/chloroform (1 1) solution at 30°C, spin-spin coupling constants ^3J of the vicinal H-NC$^\alpha$-H and H-C$^\alpha$C$^\beta$-H protons, deuterium exchange half-times $t_{1/2}$ of amide protons at 30 and 50°C, NOE d_1- and d_3-connectivities of the amide proton of residue $i+1$ with, respectively, the C$^\alpha$ and C$^\beta$ protons of the preceding residue i and chemical shifts $\Delta\delta$ exhibited by amide protons when [Cs$^+$]/[GA] molar ratio changed from 0 6 to 1 1

Amino acid residue	δ (± 0 01 ppm)				$^3J_{HNC^\alpha H}$ (±0 3 Hz)	$^3J_{HC^\alpha C^\beta H}$ (±0.3 Hz)	$t_{1/2}$ (h)		d_1	d_3	$\Delta\delta$ (± 0 01 ppm)
	NH	C$^\alpha$H	C$^\beta$H	Others			30°C	50°C			
Formyl				HCO 8 33							
L-Val 1	8 27	4.87	2.00	C$^\gamma$H$_3$ 0.98;0 87	9 0	7 5	<0 2				0 05
Gly 2	8.67	4 45;3 92					0 2		yes	yes	0 04
L-Ala 3	8.77	5.50	1 48		8.4	7 2	0.6		yes		0 01
D-Leu 4	9.20	5 21	1.95;1 95	C$^\gamma$H 1 68 C$^\delta$H$_3$ 1 17;1 10	8 8		23		yes	yes	0
L-Ala 5	9 30	4 76	1.54		6 0	7 5	<0 2		yes	yes	0
D-Val 6	8 23	5.48	2 29	C$^\gamma$H$_3$ 1 24,1 24	7.2	6.4	64	4.4	yes	yes	0 05
L-Val 7	8.55	5 76	2 17	C$^\gamma$H$_3$ 1 16,1.02	9.4	4.0	>100	5 0	yes	yes	0 04
D-Val 8	8.98	4 94	2 05	C$^\gamma$H$_3$ 1 05,0.79	8.4	6 8	>100	19	yes	yes	0 01
L-Trp 9	9.04	5 33	3 52,3 45	N1H 10 39 C2H 7.20 C4H 7.72 C5H 7.11 C6H 7 21 C7H 7 46	8 4	7 5;7.5	>100	6 7	yes	no	0 02
D-Leu 10	8.87	5.21	1.18;0 73	C$^\gamma$H 0.60 C$^\delta$H$_3$ 0 60;0 24	7 6	6 4;6.4	>100	16	yes	yes	0
L-Trp 11	9.34	5 08	3 31;3 14	N1H 10 65 C2H 7 11 C4H 7.78 C5H 7.19 C6H 7 31 C7H 7.56	9 2	4 5;10.5	>100	6.3	yes	no	−0.03
D-Leu 12	9.09	5 56	1.86;1.86	C$^\gamma$H 1 90 C$^\delta$H$_3$ 1.27;1 18	6 4	6.8;6 8	>100	4.3	yes	yes	0.05
L-Trp 13	9.76	5 37	3.50;3 33	N1H 10 52 C2H 7 16 C4H 7 51 C5H 7 13 C6H 7.26 C7H 7 49	9.6	8 3;8 3	>100	2 0	yes	no	−0 08
D-Leu 14	7.50	4.93	1 06;0 83	C$^\gamma$H 0.60 C$^\delta$H$_3$ 0 60;0 60	8.8	6.8;9.8			yes	yes	
L-Trp 15	8 57	5 21	3.43;3.20	N1H 10.32 C2H 7.06 C4H 7.72 C5H 7 09 C6H 7 26 C7H 7.49	9.6	5 3;11 3	>100	2 3	yes	no	0.04
EA	8.52	4 08,3.35	3.74;3.49				7 7		yes	no	−0 08

Additional information on the spatial structure of the Cs$^+$–GA complex was obtained from spin-spin coupling constants of vicinal H-NC$^\alpha$-H protons and from deuterium exchange of amide NH groups (table 1). The coupling constants were measured directly from the resolution enhanced 1D NMR spectrum. For deuterium exchange measurements the Cs$^+$–GA complex was lyophilized from $CD_3OH/CDCl_3$ solution and then redissolved in $CD_3OD/CDCl_3$. The exchange half-times $t_{1/2}$ were estimated from the time dependence of peak intensity of corresponding NH signals.

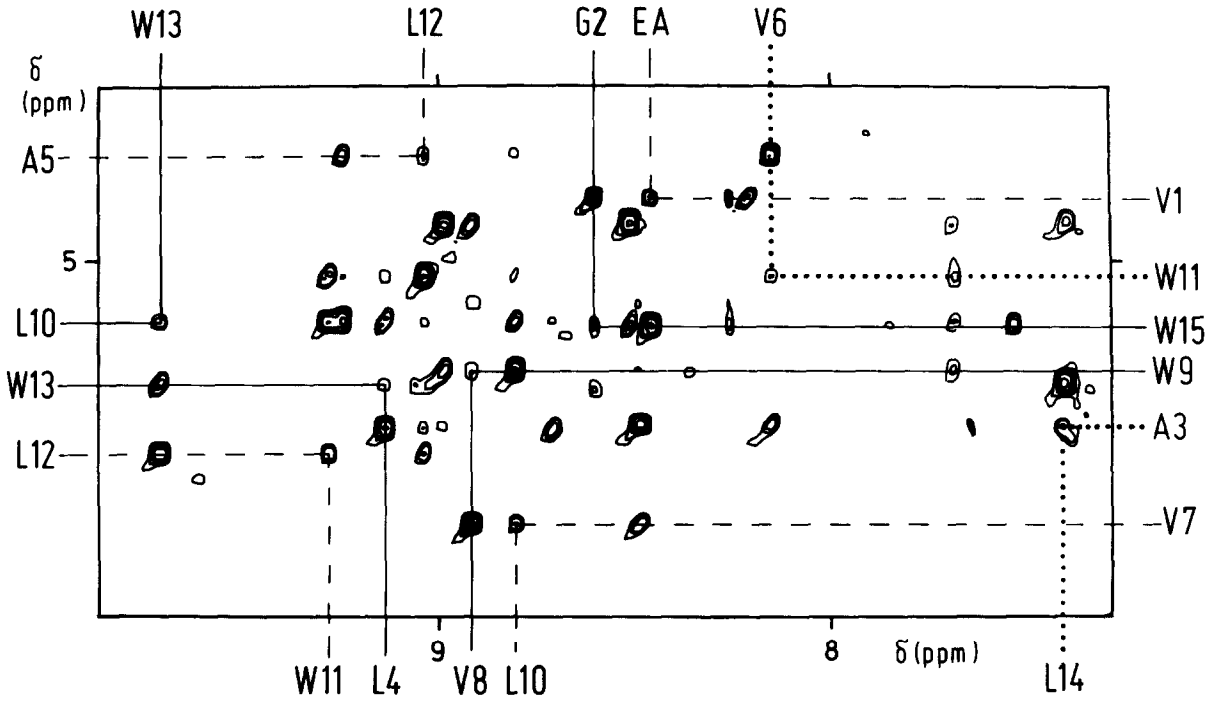

Fig.2. Contour plot of the region (ω_1 = 3 2–5 9 ppm, ω_2 = 7 3–9.9 ppm) of a phase sensitive absorption mode NOESY proton spectrum (mixing time 200 ms) of a 0 03 M [Val^1]gramicidin A complex with caesium ([Cs^+]/[GA] = 1.1) solution in $CD_3OH/CDCl_3$ (1.1) at 30°C. The digital resolution is 5 Hz/point for both ω_1 and ω_2 directions. The spectrum was recorded in 22 h The amide NH proton chemical shifts are indicated by the assignments at the top and at the bottom, those of C^α protons on the left- and right-hand sides of the plot

The majority of backbone NH groups exchange slowly enough to indicate their participation in hydrogen bonding. At both 30 and 50°C, the NH signal of D-Leu-14 is hidden in the tryptophan aromatic region and thus its $t_{1/2}$ values were not measured.

The observation (see table 1) of d_1-connectivities over the entire amino acid sequence [24,26] as well as of relatively large values of spin-spin coupling constants of vicinal H-NC$^\alpha$-H protons [20] confirms an extended conformation for the polypeptide backbone of the Cs^+–GA complex. The data on tertiary NOEs (between protons of non-adjacent residues in the amino acid sequence) unequivocally prove that the Cs^+–GA complex is formed by two extended, equivalent chains adjusted in antiparallel manner as shown in fig.3, and rolled into a right-handed $\uparrow\downarrow\pi\pi^{7\,2}_{\mathrm{L\,D}}$ double helix with 7.2 residues per turn (fig.4). In accordance with deuterium exchange of amide NH groups (table 1), the molecular structure is stabilized by 26 interchain hydrogen bonds (fig.4). The amide NH groups of Val 1, Ala 3 and Ala 5, as well as the carbonyl groups of Val 1 and Ala 3 do not participate in hydrogen bonds. Although the exchange of the Gly-2NH is also rapid, nevertheless, according to the NOE connectivities and overall molecular structure it is highly likely that this NH amide group participates in interchain hydrogen bonding.

The $\uparrow\downarrow\pi\pi^{7\,2}_{\mathrm{L\,D}}$ dimer is 2.7 nm long (as estimated by the distance between the Val 1 nitrogens) and has a C_2 symmetry axis perpendicular to the helix axis. The conformation appears to be reasonably rigid as far as the peptide backbone is concerned, but the side chains are subject to a considerable degree of flexibility as manifested by H-C$^\alpha$C$^\beta$-H proton-coupling constants (see table 1). The central axial cavity, which has a luminal diameter of ~0.4 nm (as estimated from the distance between

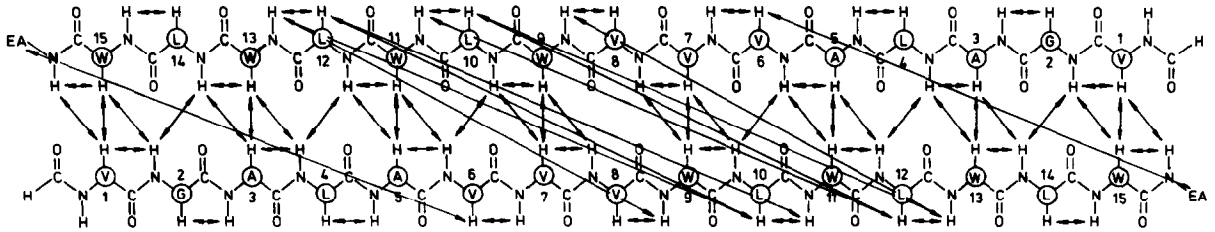

Fig.3 Intra- and interchain NOE-connectivities between backbone protons of Cs^+–[Val^1]gramicidin A complex Some of the connectivities which are expected according to the deduced spatial structure of the complex (fig 4) were not revealed because the positions of corresponding cross-peaks interfere with strong diagonal band or overlap with other cross-peaks.

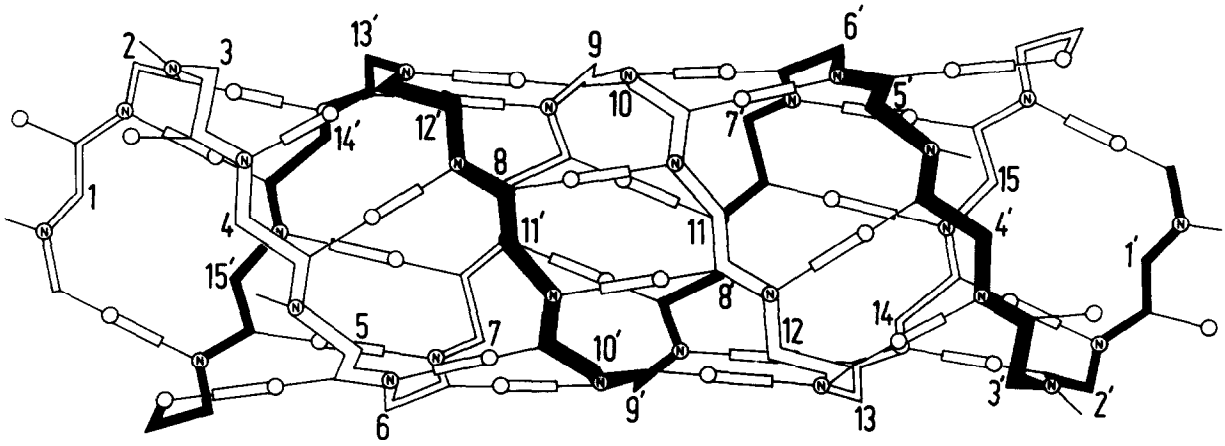

Fig.4 Schematic presentation of the right-handed double-helix of gramicidin A complex with caesium Side chains are omitted for clarity. Interchain hydrogen bonds are shown by empty bars

the carbonyl oxygens and the helix axis minus the van der Waals radius of the carbonyl oxygen), is lined by polar peptide groups, while the nonpolar side chains project from the exterior surface of the dimer. This arrangement in principle permits the molecules to be incorporated into lipid bilayer membranes and form water-permeable cation-selective channels. The IR amide I band at 1631 ± 1 cm^{-1} of Cs^+–GA in methanol/chloroform is in the frequency range 1628–1635 cm^{-1} of GA in liposomes [3,28–30].

The dimension of the internal cavity of the $\uparrow\downarrow\pi\pi^{7,2}_{LD}$ dimer is better suited to accommodate the caesium cation (0.33 nm ion diameter), which demonstrates the highest permeability via GA channels in bilayer lipid membranes among alkali metal cations [27], than the other double helical structures of GA directly demonstrated by 2D NMR spectroscopy – $\uparrow\downarrow\pi\pi^{5,6}_{LD}$ [19,20] and $\uparrow\uparrow\pi\pi^{5,6}_{LD}$ [21] which have only ~0.3 nm diameter of the axial cavity.

In conclusion, we would like to mention that this study again directs attention to the double helical structure of the GA transmembrane channel [3,10,16,19–21,30] in addition to the more widely accepted single helical head-to-head dimer [4,9,11,28,29,31]. Both double-stranded $\uparrow\downarrow\pi\pi^{7,2}_{LD}$ and single-stranded $\overrightarrow{\pi^{6,3}_{LD}}\overleftarrow{\pi^{6,3}_{LD}}$ helical structures have similar dimensions, including the diameter of the axial cavity, and similar arrangement of hydrophobic and hydrophilic surfaces. They are indistinguishable by conformational energy calculations [17,18] and by X-ray study of crystals [15]. The final decision between these alternatives must await direct elucidation of the conformations of the GA dimer and its complex with alkali metal cation in membranes.

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