2D NMR OF SINGLE AND DOUBLE STRANDED HELICES OF GRAMICIDIN A IN MICELLES AND SOLUTIONS

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INTRODUCTION

The linear pentadecapeptide gramicidin A (GA) HCO-*L*-Val^1-Gly^2-*L*-Ala^3-*D*-Leu^4-*L*-Ala^5-*D*-Val^6-*L*-Val^7-*D*-Val^8-*L*-Trp^9-*D*-Leu^{10}-*L*-Trp^{11}-*D*-Leu^{12}-*L*-Trp^{13}-*D*-Leu^{14}-*L*-Trp^{15}-NHCH$_2$CH$_2$OH attracts attention due to its ability to form monovalent cations permeable channels in membranes. Once it has been appreciated (1), that two molecules of GA are combined in such a way as to span the membrane bilayer (Fig. 1) a number of detailed models of the channel were proposed. The models were based on obvious requirements of such a structure: (i) a lipophilic exterior and hydrophilic axial cavity; (ii) a length sufficient to span the lipid portion of the bilayer; and (iii) the capacity to interact with ions. The problem of spatial structure of the GA channel is dramatically complicated because of diversity of low-energy conformations, which are promoted by alternation of the *L*- and *D*-amino acid residues (2). Among them are single (3,4) and double (5) stranded helices with different relative orientation of the N- and C-terminals, differen handedness, and different number of residues per turn. In principle most of the conformations might be considered as potential channel formers, as was substantiated by indirect evidences (5-12).

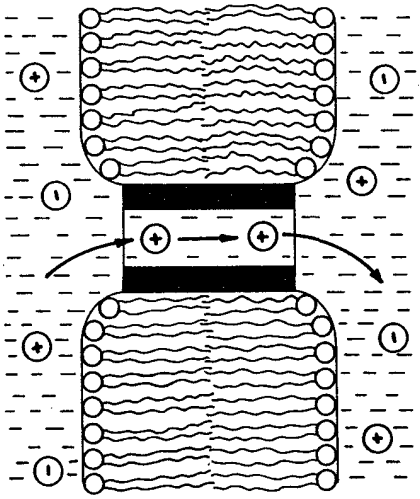

Figure 1. Schematic illustration of a gramicidin A channel in a lipid bilayer membrane.

This report covers our recent results of the NMR study of a variety of GA conformations in organic solvents and in membrane environment (13-20). High resolution NMR of membrane-bound peptides and proteins are usually limited to artificial milieus, which mimic native membranes and provide suitable NMR spectra. Thus two different types of milieus were examined (i) organic solvents and (ii) micelles. The NMR studies in organic solvents are of substantial interest, even though the CD technique indicates that GA adopts different conformations in solutions and in membrane (21). Firstly, once a conformation in organic solvent is unambiguously determined, it can be ruled out as a candidate for channel state of GA in lipid bilayers. Secondly, a comparison of the GA conformations in organic solvents and in membranes is essential to get molecular insight into the modulation of the peptide structure by membranes. The highlight of this work is elucidation of GA conformation in membrane environment, which was provided by SDS micelles. It is demonstrated that the mi-

celles create the ion-channel state of GA and ensure high resolution NMR spectra.

CONFORMATIONS OF GRAMICIDIN A IN SOLUTION

In organic solvents GA forms an equilibrated mixture of at least four conformers called species 1, 2, 3 and 4 (5) with so slow interconversion rate that they are observed separately in the 500 MHz spectrum (Fig. 2a). Conformational NMR analysis of those species might be greatly assisted if they are individually isolated. After crystallization from ethanol at 20°C as in (5) and subsequent dissolving in dioxane-d_8, the ^{1}H NMR COSY spectrum of the 10 mM solution GA (Fig. 2b) was found to correspond to the single species 3 conformer (13,14). The conformer has one signal for each chemically equivalent proton.

Proton signal assignment to specific position in the amino acid sequence was carried out in two steps. The spin systems of all amino acid residues were identified by analysis of the COSY and in some difficult cases by analysis of the RELSY spectra (13,14). Spin systems of the NH-C$^\alpha$H-C$^\beta$H$_2$ protons of tryptophan residues were linked to spin systems of indole protons through NOE-connectivities of the C$^\beta$H protons with the C(4)H and C(2)H ring protons. The next step was the assignment of a spin system to specific position of the amino acid residue in the primary structure of the peptide. This was done by the NOESY spectra sequential resonance assignment through the $^1d_{\alpha N}$, $^1d_{NN}$ and $^1d_{\beta N}$ NOE-connectivities for amide $N_{i+1}H$ proton of residue i+1 with, respectively, the $C^\alpha_i H$, $N_i H$ and $C^\beta_i H$ protons of the foregoing residue i as in (22). The assignment of the species 3 backbone resonances was based on the $^1d_{\alpha N}$ connectivities summarized in Fig. 3c. The pattern of the ^{1}d-connectivities (all $^1d_{\alpha N}$ were observed, whereas no $^1d_{NN}$ was revealed) and relatively high values of the vicinal spin-spin coupling constants H-NC$^\alpha$-H (13,14) indicate that polypeptide backbone of species 3 has an extended conformation. In addition, analysis of NOESY spectra reveals over 50 through space connectivities between residues which are distant in the amino acid sequence (13,14). The concise map of NOE-connectivities

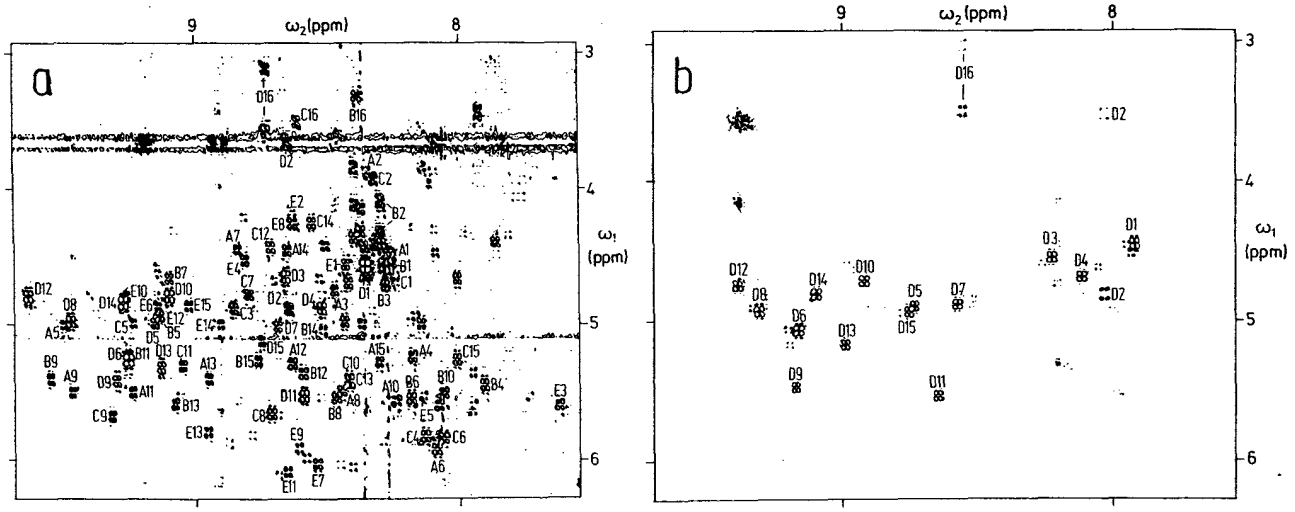

Figure 2. Spectral regions of a 500 MHz phase-sensitive absorption mode DQF-COSY spectra of gramicidin A solutions at 30°C: (a) 30 mM gramicidin A in CD_3CD_2OH; (b) 10 mM gramicidin A species 3 solution in dioxane-d_8. The cross-peaks of NH-C$^\alpha$H protons are identified with letters A (species 1), B and C (two unsymmetrically aligned chains of species 2), D (species 3) and E (species 4) and number in the amino acid sequence of gramicidin A.

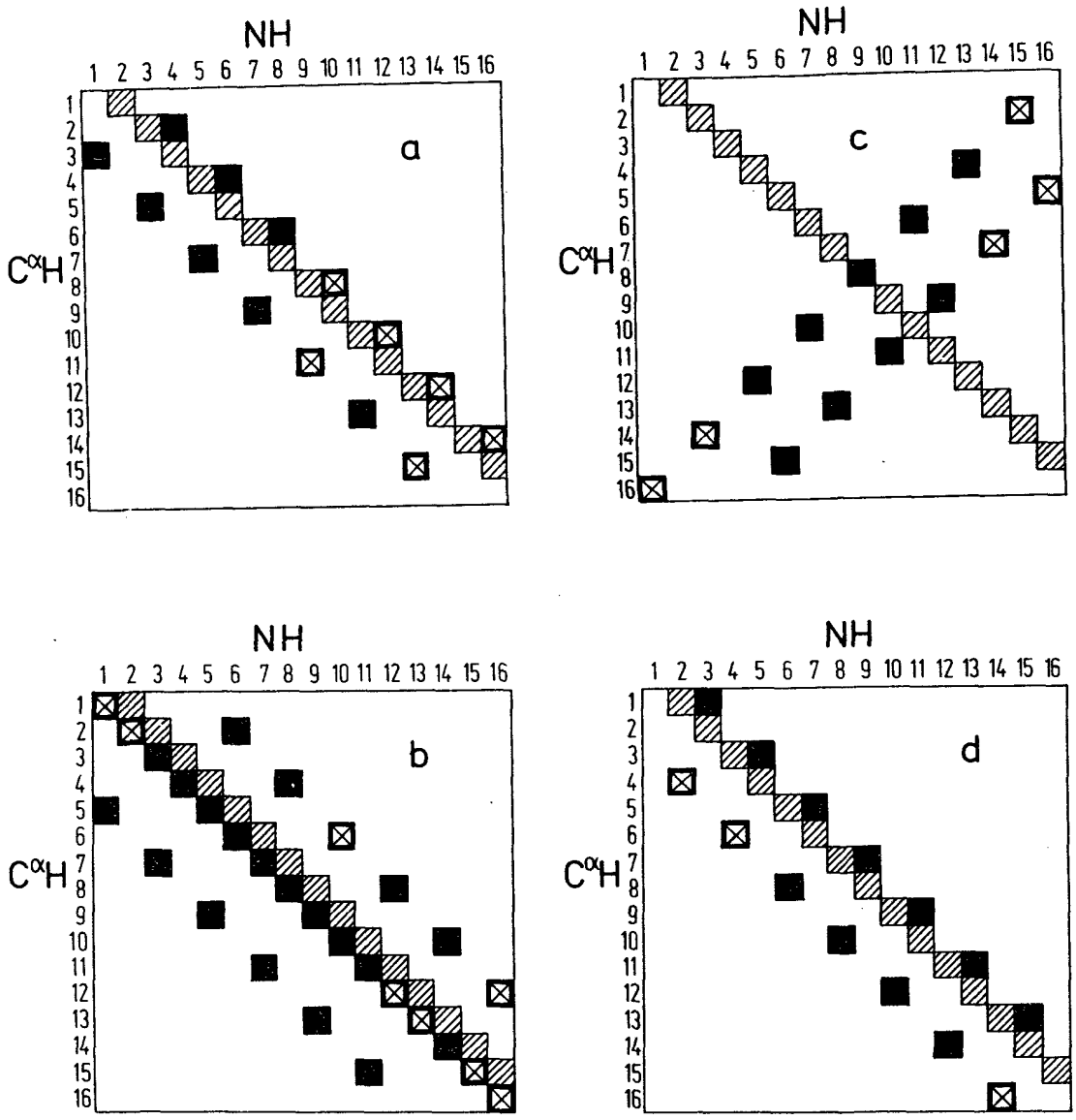

Figure 3. Schematic maps of NOE-connectivities between backbone NH and $C^{\alpha}H$ protons of the gramicidin A species 1, 2, 3, and 4 [(a), (b), (c) and (d), respectively]. The filled and crossed squares correspond, respectively, to the identified and ambiguous NOE cross-peaks for dimer interchain connectivities; the hatched squares correspond to the identified intrachain connectivities ($^{1}d_{\alpha N}$).

(Fig. 3c) clearly demonstrates the dimeric nature of species 3. An important aspect of the polypeptide spatial structure is the intra- and inter-molecular hydrogen bonds. They were revealed by the rate of deuterium exchange of NH protons with solvent. The majority of amide NH groups of species 3 slowly exchange with deuterium when 4% CD_3OD is added to dioxane-d_8 solution indicating their participation in hydrogen bonding. Only the NH protons of Val^1 and Gly^2 residues exchange somewhat faster (14). To fit the spatial structure of the species 3 with the experimental NMR data, two equivalent extended polypeptide chains have to form an antiparallel β-structure, which is rolled into left-handed double helix $\uparrow\downarrow\pi\pi^{5.6}_{LD}$ with 5.6 residues per turn as shown in

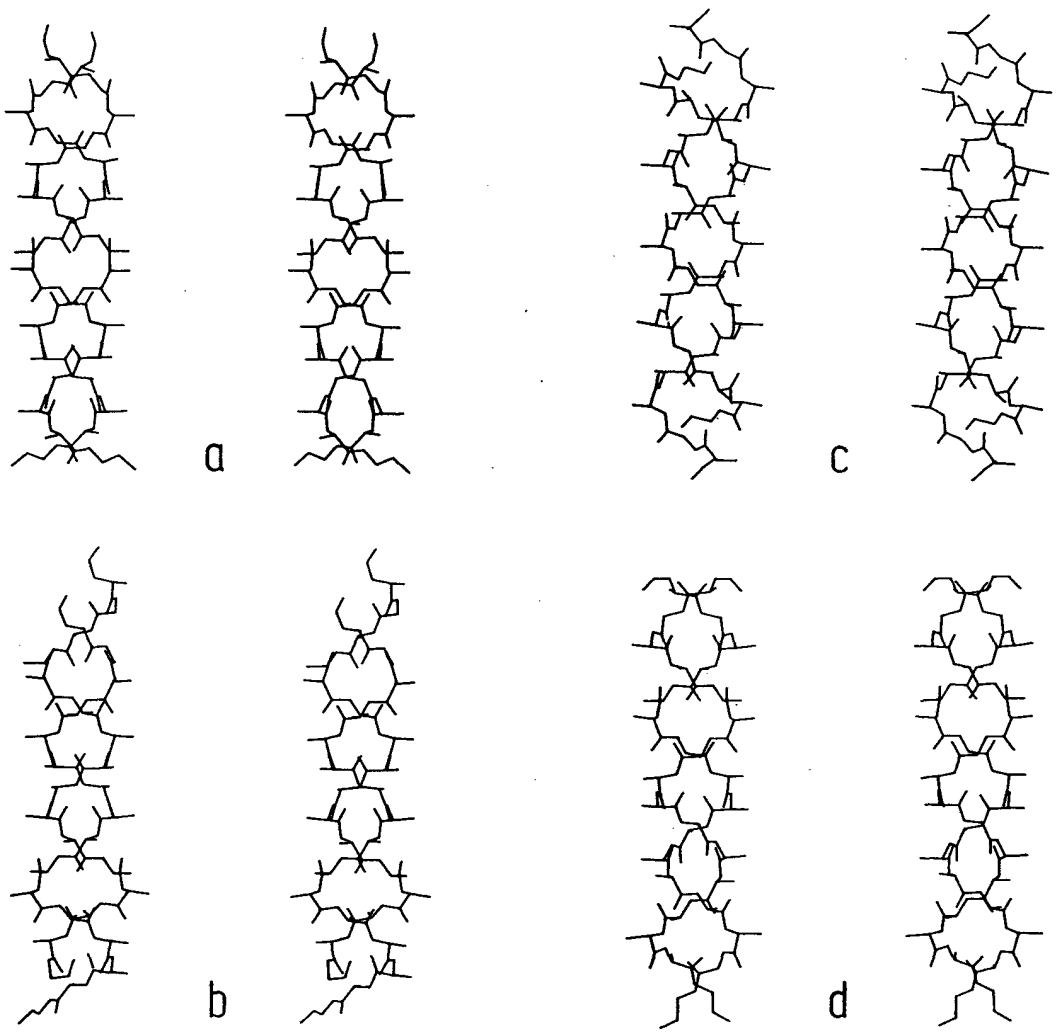

Figure 4. Stereo drawing of the gramicidin A double helices observed in organic solvent. (a) Species 1 - left-handed parallel helix $\uparrow\uparrow\pi\pi^{5}_{LD}{}^{6}$; (b) Species 2 - left-handed parallel helix $\uparrow\uparrow\pi\pi^{5}_{LD}{}^{6}$ with unsymmetrical alignment of backbones; (c) Species 3 - left-handed antiparallel helix $\uparrow\downarrow\pi\pi^{5}_{LD}{}^{6}$; (d) Species 4 - right-handed parallel helix $\uparrow\uparrow\pi\pi^{5}_{LD}{}^{6}$, a mirror image of species 1. Side chains but C^{β} atoms are omitted for clearer presentation.

Fig. 4c. It has to be noted that this conformation comprises a few intermolecular NOE-connectivities marked in Fig. 3c by crossed squares, which cannot be revealed because the positions of corresponding cross-peaks are too close to a strong ridge of the NOESY spectrum.

Other conformational forms of GA in organic solvent are not easy to isolate in enough for NMR study quantity. Thus to imitate species 4 use was made of the synthetic shortened derivative - des[*L*-Ala^3, *D*-Leu^4, *L*-Ala^5, *D*-Val^6]-gramicidin A, CD and IR spectra of which are similar (23) to those of species 4. All signals in the NMR spectra of the derivative were assigned by COSY, NOESY, RELSY, as well as in complicated case by double-quantum 2D NMR and RELAY-NOESY techniques (15). The NOE-connectivity map combined with other NMR results (spin-spin coupling constants of H-NC$^{\alpha}$-H protons and deuterium exchange rates of NH protons) lead to the conclusion, that the conformation of the shortened derivative is a right-handed parallel double helix

$\uparrow\uparrow\pi\pi^{5.6}_{LD}$ with 5.6 residues per turn (15).

Taking into account the above results, that are assigned 2D NMR spectra and spatial structure of species 3, and spatial structure of species 4, we finally resolve and identify the spectral components in the mixture of four conformational forms that present in organic solvent. The COSY spectrum in Fig. 2a shows that in addition to the four species there is at least one minor component conformation of which we failed to settle. Analysis of the NMR experimental data (e.g., see Fig. 3a-d), obtained by various sorts of 2D techniques (COSY, NOESY, RELAY-NOESY etc.) reveals that conformations of species 1-4 are the following (Fig. 4): the species 1 - parallel left-handed double helix with symmetrical allignment of the backbones; the species 2 - also parallel left-handed double helix, but with unsymmetrical alignment of the backbones, each of which gives different signals in the NMR spectra; the most abundant species 3 - antiparallel left-handed double helix, the structure of which was analysed in full details separetely; and the least abundant species 4 - parallel right-handed double helix, a mirror image of species 1. All of the double helices have 5.6 residues per turn.

Thus the problem of the GA conformations in organic solvents is now resolved. It should be noted that general features of the double helical structures of GA in organic solvents were predicted on the basis of CD and IR spectra analysis (5). The results from the NMR study, correlated with CD data on GA in organic solvents and in vesicles (5,21), lead us to the conclusions that (i) double helices with 5.6 residues per turn (Fig. 4) must be ruled out as a channel state conformations, (ii) organic solvent alone does not mimic GA environment in membranes. But as ionic channel is concerned, one might expect a channel state of GA is generated by a joint effect of organic solvent and channel permeable cations.

COMPLEX GRAMICIDIN A WITH CESIUM

Addition of lithium and sodium salts to the solution of GA in methanol-chloroform (1:1) mixture (the composite solvent is idealy suited for preparation of high concentration of a GA complex) induces formation of random coil conformation, as for free peptide in dimethylsulfoxide. Contrary, ions with larger diameters, as potassium, thallium and cesium, form stable complexes with GA possessed definite structure.

With addition of cesium thiocyanide to the solution of GA, the intensity of signals arised from the complexed form are growing until the plateau is riched at equimolar metal-GA ratio (Fig. 5). This is due to two-site binding reaction, in which the first metal binding step is a slow process, while the second step is fast in the NMR time scale (16). The affinity for the second cation binding is lower than for the first cation, presumable due to electrostatic repulsion between

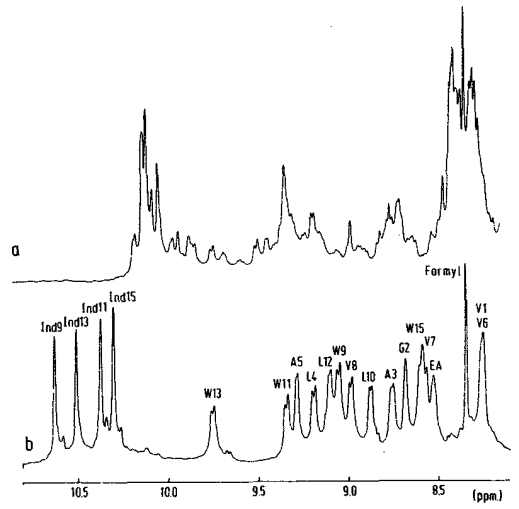

Figure 5. Low field region of 500 MHz 1D NMR spectra of a 30 mM solution of gramicidin A in CD_3OH-$CDCl_3$ (1:1), 30°C (a) without cesium salt; (b) with $[Cs^+]/[GA]$ molar ratio of 1.1. The backbone NH resonance assignments are indicated by one-letter code for the amino acid residues and the serial number in primary structure. EA - amide proton of ethanolamine moiety; Ind - indole NH proton of tryptophan residues.

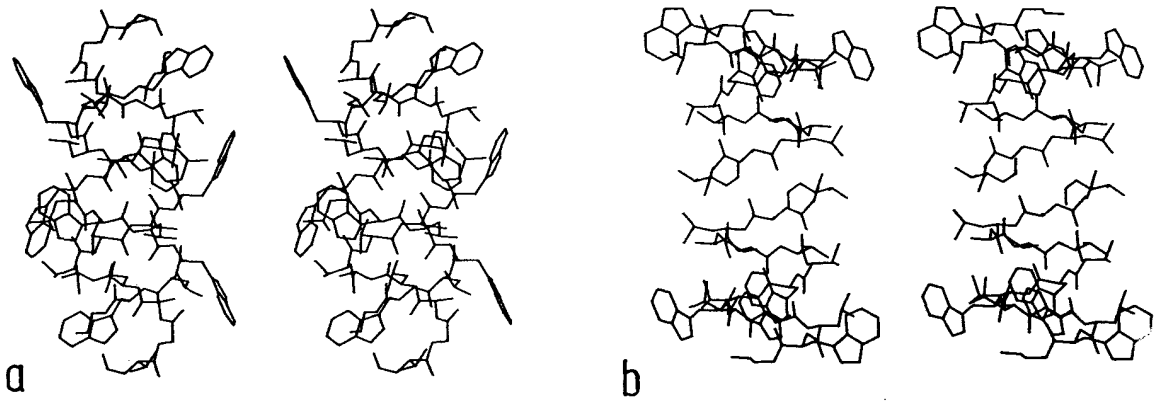

Figure 6. Stereo drawing of gramicidin A conformations. (a) Complex of gramicidin A with cesium in organic solvent - right-handed antiparallel double helix $\uparrow\downarrow\pi\pi^{7}_{LD}{}^{2}$. All side chains of the structure are highly mobile, so one of the conformations is shown. (b) Gramicidin A incorporated into SDS micelle - right-handed single-stranded helix $\overrightarrow{\pi}^{6}_{LD}{}^{3}\overleftarrow{\pi}^{6}_{LD}{}^{3}$, side chains of all residues, but Val^{7} and *D*-Leu^{4}, have unique conformations.

cations. All proton signals of GA-cesium complex were assigned by 2D NMR techniques. Combining NMR parameters that are J-couplings, deuterium exchange rates of amide protons and NOE-connectivities between distinct protons, we arrived at the right-handed antiparallel double helix $\uparrow\downarrow\pi\pi^{7}_{LD}{}^{2}$ with 7.2 residues per turn (Fig. 6a) (16). The conformation appeares to be reasonable rigid as far as the peptides backbone is concerned, but the side chains are subjected to a considerable degree of flexibility as manifested by H-$C^{\alpha}C^{\beta}$-H proton coupling constants and by conflicting set of NOE-connectivities, which is impossible to fit to unique fixed conformations of side chains. The $\uparrow\downarrow\pi\pi^{7}_{LD}{}^{2}$ dimer is ~2.6 nm long and has a C_2 symmetry axis perpendicular to the helix axis. The central axial cavity, which has a luminal diameter of ~0.4 nm, 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 molecule to be incorporated into lipid bilayer membranes and to form water-permeable cation-selective channel. The dimensions of the internal cavity of the $\uparrow\downarrow\pi\pi^{7}_{LD}{}^{2}$ dimer is better suited to accomodate the cesium cation, which demonstrates the highest permeability via GA channels in membranes among alkali metal cations (24), than other double helical structures of GA that has been found in organic solvents. However, comparison of the CD spectra for the GA complex with cesium and for GA incorporated into vesicles shows that conformations in the milieus are different (21). Thus the combined effect of organic solvent and cations cannot force GA to take a conformation of lipid bilayer ion-channel. Again, the $\uparrow\downarrow\pi\pi^{7}_{LD}{}^{2}$ double helix has to be excluded as a candidate for actual ion-channel state.

ION-CHANNEL CONFORMATION OF GRAMICIDIN A

GA, which is practically insoluble in water, was incorporated into micelles by addition of its 0.05 M solution in trifluoroethyl alcohol-d_3 into a 0.5 M sodium dodecyl-d_{25} sulphate (SDS-d_{25}) dispersion in water. The sample was diluted with water to final concentration of 5 mM of GA and 250 mM of SDS-d_{25} in water-trifluoroethyl alcohol (molar ratio 16/1) medium. These experimental conditions correspond to incorporation of not more than two peptide molecules into one micelle. The CD spectrum of GA in SDS micelles is practically identical to that of GA in dipalmitoyl phosphatidylcholine liposomes (Fig. 7). The red shift of the liposome spectrum is ascribed to light scattering of lipid suspensions (see (25)).

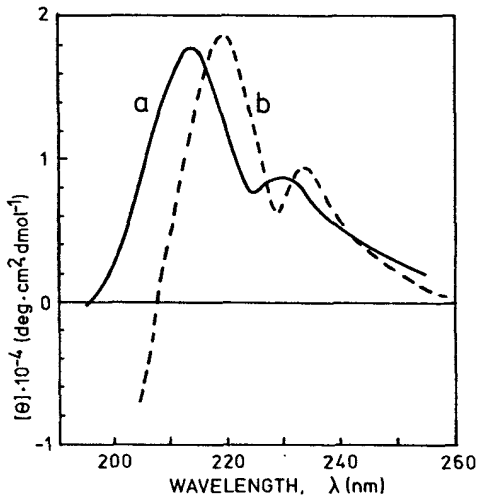

Figure 7. CD spectra of (a) 5 mM Gramicidin A incorporated into SDS micelles in water-trifluoroethanol solution and (b) 0.5 mM gramicidin A incorporated into dipalmitoyl phosphatidylcholine liposomes (peptide/lipid molar ratio of 1/330) after heat incubation, 15 hr at 70°C.

The unique CD pattern has been universally accepted as proof that the system contains a channel state of GA (11,26). Furthermore, the ^{23}Na NMR spectra prove that GA embeded into SDS micelles does

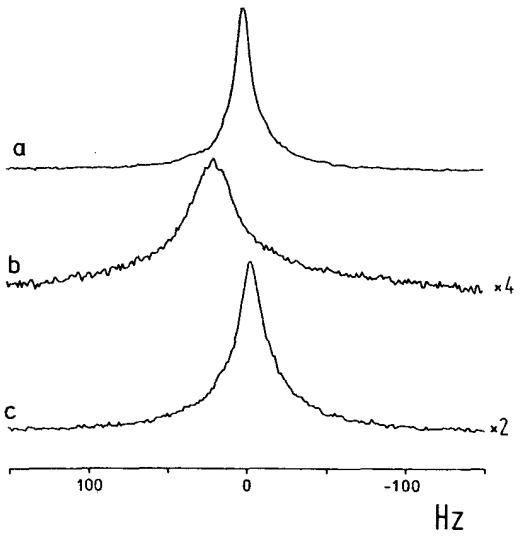

Figure 8. Sodium-23 nuclear magnetic resonance spectrum at 132 MHz, 55°C and pH 6.5. (a) 250 mM solution of SDS in water-trifluoroethanol (molar ratio of 16/1); (b) as above but 5 mM gramicidin A was added; (c) with 30 mM solution of $TlClO_4$ in sample (b).

indeed interact with alkali metal ions (Fig. 8a,b) and the interactions are competitively blocked by Tl^+ ions (Fig. 8c), which also competitively block alkali metal ion transport through the channel in lipid bilayer membranes (27,28). Therefore, one remains to conclude that GA in SDS micelles forms an ion-channel with a similar molecular structure to that in lysolecithin and in liposomes.

The proton NMR spectra of GA incorporated into SDS-d_{25} micelles (Fig. 9) display features, which make them acceptable for high resolution 2D NMR study of the detailed molecular structure. It should be specifically emphasized, that the system in SDS contains an abudance of sodium cations relative to the GA concentration, thus the results to be discussed correspond to the ion-channel inflated by sodium

Chemical shifts of all protons were assigned to specific positions in the amino acid sequence of GA by conventional procedure of sequential resonance assignments (17,18). Fig. 9a demonstrates assignments in a fingerprint region (J-cross-peaks of H-NC$^\alpha$-H protons) of the COSY spectrum. The 2D NOESY spectra reveals almost 200 NOE-connectivities between distinct protons (18). The most illustrative data for molecular conformation analysis are presented in Fig. 9. On the one hand, the NOE-connectivities between the $N_{i+1}H$ and C^α_iH protons (see Fig. 9b) being in combination with relative high (>9 Hz) values of vicinal backbone H-NC$^\alpha$-H proton spin-spin couplings (measured in COSY spectrum, Fig. 9a) lead us to conclude that GA has an extended conformation. On the other hand, the NOE-connectivities between the N_iH and $C_{i+6}H$ protons of *D*-residues and between the C_iH and $N_{i+6}H$ protons of *L*-residues (Fig. 9b) as well as slow exchange rates observed for all but the *D*-Leu^{12,14} and C-terminal ethanolamine NH protons suggest that the extended backbone is spiraled. Taken together, these data inherently produce a right-handed single-stranded $\pi^6_{LD}{}^3$ helix with 6.3 residues per turn. The alternative left-handed helix proposed by Urry et al. (3,11) for the same CD pattern of GA (Fig. 7) is excluded from further

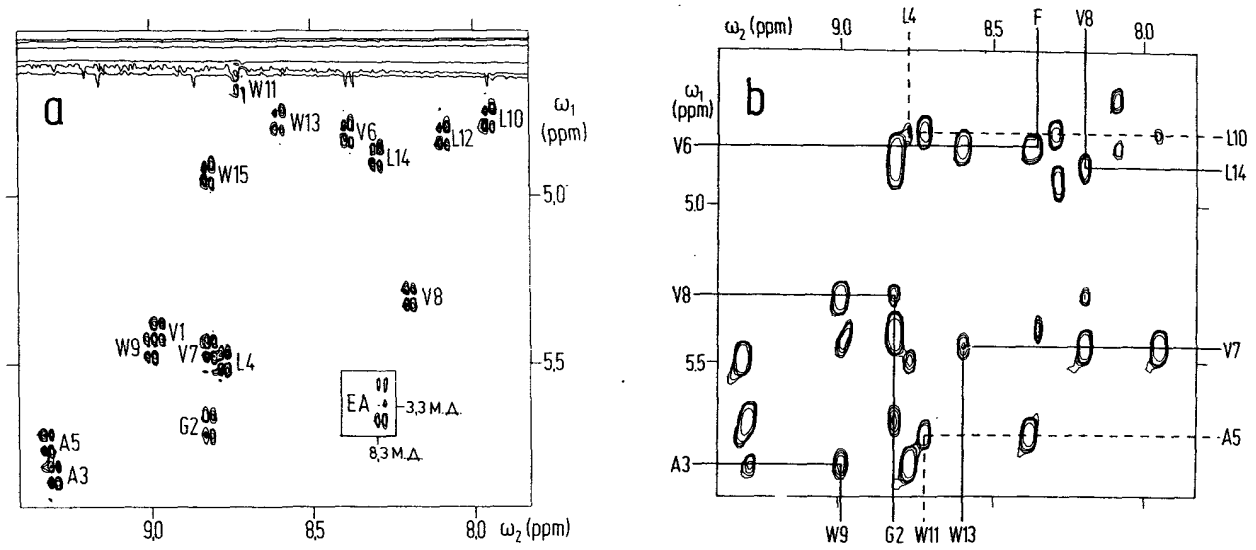

Figure 9. Spectral regions of the NH - $C^{\alpha}H$ protons of a 500 MHz phase-sensitive absorption mode 2D-NMR spectra of 5 mM gramicidin A in SDS-d_{25} micelles (peptide/detergent molar ratio of 1/50) in H_2O-CF_3CD_2OD (molar ratio of 16/1) at pH 6.5 and 55°C. (a) COSY spectrum. Assignments of HN - $C^{\alpha}H$ cross-peaks are indicated by one-letter code. (b) NOESY spectrum (mixing time 100 ms). The amide N_iH proton chemical shifts are indicated by the assignments at the top and the bottom, those of $C^{\alpha}_{i+6}H$ (*D*-residues) and $C^{\alpha}_{i-6}H$ (*L*-residues) protons on the left- and right-handed sides of the plot.

consideration as being inconsistent with the NOE-connectivities and deuterium exchange rates of the NH protons.

Some of the observed NOE-connectivities (e.g.: NH(Val^1)...NH(Ala^5), NH(Val^1)... $C^{\beta}H_3$(Ala^5) and $C^{\alpha}H$(Gly^2)... $C^{\alpha}H$(*D*-Leu^4)) and slow exchange rates of NH protons of Val^1 and Ala^{3,5} cannot be rhymed with monomer molecule, so they were fit to the dimer conformation that is N-terminal-to-N-terminal right-handed single-stranded $\overrightarrow{\pi}^{6.3}_{LD}$ $\overleftarrow{\pi}^{6.3}_{LD}$ helix. The dimer has symmetry axis perpendicular to the channel axis in accordance with NMR observation of only one signal for each chemically equivalent proton. The structure is stabilized by 20 intramolecular and 6 intermolecular hydrogen bonds NH...O=C.

The corresponding wire model was constructed and side chains of amino acid residues were adjusted in accordance with the vicinal H-$C^{\alpha}C^{\beta}$-H proton couplings and NOE-connectivities of side chain protons. The torsion angles ϕ, ψ, χ^1 and χ^2 measured on the model were used as initial those for conformational energy optimization (19).

Conformational energy optimization was done using fixed bond angles and bond lengths and planar rigid trans-peptide units. Energy parameters and geometry of amino acid residues were identical to those used in ECEPP/2 (29,30). The energy refined conformation was made use as a basis for exhaustive search of conformations which are in agreement with NMR data. Torsion angles ϕ, ψ, χ^1 and χ^2 of amino acid residue, one after the other, were modified in a systematic way and after energy minimization each conformation was compared with NMR data set. For this purpose, a special computer program has been developed (19). The program uses as an input the list of NMR parameters - NOE cross-peaks, spin-spin coupling constants of vicinal protons and solvent accessibility of NH groups. The output of the program is the NOE cross-peaks assignment, stereochemical assignment of NMR signals and list of violated spectral parameters. Analysis of the output lead us to conclude that backbone conformation of GA incorporated into micelles is unequivocally defined by NMR data, except that of the

Gly^2-Ala^3 peptide bond orientation and highly mobile C-terminal ethanolamine group. Side chain conformations of all amino acid residues, except of the angles χ^1 of Val^7 and χ^2 of *D*-Leu^4 residues, are located in corresponding single potential wells. One of the low energy conformations which is in exellent accordance with the NMR data set is shown in Fig. 6b.

It is of interest to compare structures of the GA complex with cesium (Fig. 6a), observed in organic solvent (16), and of the GA ion-channel (Fig. 6b), observed in micelles (17-20). Both helices have hydrophobic external surfaces and polar internal axial cavities. What's more, the dimensions of this helices are the same - axial cavity diameter of ~0.4 nm and axial cavity length of ~2.6 nm. The question is why GA adopts $\uparrow\downarrow\pi\pi^7_{LD}{}^2$ helix in isotropic organic solvent and $\overrightarrow{\pi}^{6.3}_{LD}\overleftarrow{\pi}^{6.3}_{LD}$ helix in anisotropic micelle milieu. The double helix has almost uniform distribution of different types of amino acid residues (valines, leucines, tryptophanes) on the surface. Quite the contrary, the $\overrightarrow{\pi}^{6.3}_{LD}\overleftarrow{\pi}^{6.3}_{LD}$ helix has obvious (Fig. 6b) anisotropy of its molecular surface which fit to anisotropy of a micelle. Indole rings of tryptophan residues are located near the edges of the $\overrightarrow{\pi}^{6.3}_{LD}\overleftarrow{\pi}^{6.3}_{LD}$ dimer and they are oriented in such manner as to provide optimal interactions of their dipoles with negatively charged lipid-water interface.

To address the problem of the detailed conformation-mobility-function relashionships of the GA ion-channel, it is interesting to note that carbonyl groups of the *D*-$Leu^{10,12,14}$ residues which are located at the channel entrance form a "funnel" (Fig. 6b), where cations should be partially dehydrated on their passage across the channel. Enegry refinement demonstrates (19) that changes in side-chain and ethanolamine moiety conformations are accompanied by changes in orientation of peptide groups lining the cation pathway. A forementioned mobility of Val^7 (χ^1) and *D*-Leu^4 (χ^2) side chains, and ethanolamine group may be rationale for dispersity of ion conductivity of GA transmembrane channel. Indeed, it was recently shown (31), that replacement of isopropile group of Val^7, which modulates the interaction of carbonyl groups with cations, in GA on methyl group in [*L*-Ala^7]-gramicidin A leads to a dramatic reduction in the dispersity of conducting states of the channel.

The suitable for NMR spectroscopy artificial milieu, where actual transmembrane ion-channel conformation exist, opens a way to study site-directed spatial structure modifications with the aim to correlate the structure and function changes. On this way, spatial structure of covalently linked GA dimer, succinyl-bis(desformyl)gramicidin A, incorporated into SDS-d_{25} micelles was studied. A spatial structure of this analog is similar to those of GA, but has higher temperature stability. As one can deduce from Fig. 10b, in case of the analog there is no NH proton signal broadening up to 90°C, all resonances even become narrower due to lowering of a solution viscosity. In contrast to that, the structure of GA itself remains stable only till ~60°C (Fig. 10a). The observation is in accordance with longer mean transmembrane channel lifetime of the covalently linked analog (32).

Ion-selective membrane channels are intimately involved in virtually all the phenomena of neurophysiology. One of the fundamental questions of interest is the origin of ion selectivity. The GA channel is inpermeable to anions and divalent cations and yet it exhibits selectivity among monovalent cations with permeability sequence $H^+ > NH_4^+ > Cs^+ > Rb^+ > K^+ > Na^+ > Li^+$ (24). In principle the selectivity might be due to changes of the GA conformation. But our NMR results reveal no prononced influence of a sort of a complexed monovalent cation (Li^+, Na^+ and Tl^+) on the spatial structure of GA incorporated into micelles (18). Hence the same GA molecular model right-handed dimer $\overrightarrow{\pi}^{6.3}_{LD}\overleftarrow{\pi}^{6.3}_{LD}$ has to be used for theoretical computations of factors responsible for interactions of different ions with the peptide.

In summary, using a two-dimensional ^1H NMR spectroscopy a variety of double stranded helices of GA were prooved to

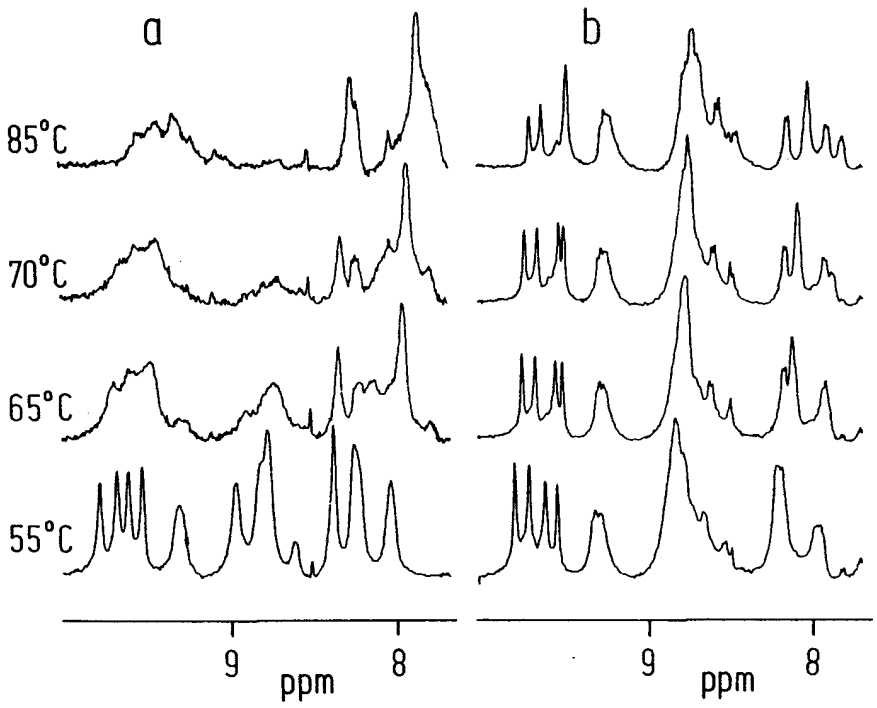

Figure 10. Low-field region of 500 MHz ^1H NMR spectra of (a) 5 mM gramicidin A and (b) 5 mM succinyl-bis(desformyl)gramicidin A in SDS-d_{25} micelles (peptide/detergent molar ratio of 1/50) in H_2O-CF_3CD_2OD (molar ratio of 16/1), at pH 6.5 and at different temperatures: 55, 65, 70 and 85°C.

exist in organic solvents. On the contrary, GA being incorporated into dodecylsulphate micelles as well as into vesicles, liposomes and black lipid membranes adopts a conformation of a single-stranded right-handed $\overset{\rightarrow}{\pi}^{6}_{LD}{}^{3}$ $\overset{\leftarrow}{\pi}^{6}_{LD}{}^{3}$ helix. The conformation of GA has been the subject of studies in a number laboratories for many years. Now, by means two-dimensional NMR techniques a deeper insight into intimate mechanism of GA ion-channeling was gained.

In the future comparison of the structures of the GA analogs with their modified ion-conducting properties should provide a more information on structure-function relationships of ion-channels.

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