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The conformation of linear gramicidin is sequence dependent

A monolayer and infrared study

N. Van Mau [1], B. Bonnet [2], A. Benayad [3], F. Heitz [1]

[1] CRBM, CNRS/INSERM, 1919 route de Mende, B.P. 5051, F-34033 Montpellier Cédex, France
[2] LAMMI, UM II, Place Eugène Bataillon, F-34095 Montpellier Cédex, France
[3] LPCSP, CNRS, B.P. 5051, F-34033 Montpellier Cédex, France

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Abstract. A comparative monolayer and infrared study of analogues of gramicidin A containing either tyrosines or naphthylalanines instead of tryptophans indicates that the nature of the aromatic residues influences the favoured conformation of the peptides. Polar residues favour the single stranded Π_{DL} helix while non polar residues favour the double stranded helix. For partly tryptophan to naphthylalanine substituted analogues the positions of the substitutions orientate the favored conformation. The nature of these substitutions may also modify the peptide-lipid interactions.

Key words: Linear gramicidins – Monolayers – Infrared spectroscopy – Conformational analysis

Introduction

From the conformational point of view, the antibiotic gramicidin A (GA) (HCO-Val[1]-Gly[2]-Ala[3]-DLeu[4]-Ala[5]-DVal[6]-Val[7]-DVal[8]-Trp[9]-DLeu[10]-Trp[11]-DLeu[12]-Trp[13]-DLeu[14]-Trp[15]-NHC_2H_4OH) (Sarges and Witkop 1965) is an extremely versatile molecule, very probably due to the presence of the D–L sequence. Indeed, slight modifications of the medium in which the peptide is dissolved can induce formation of either single (Urry 1971) or double (Veatch et al. 1974) stranded helices designated hereafter as monomer and dimer respectively, and/or interfere with the monomer–dimer equilibrium. This propensity to adopt various helical conformations was confirmed by crystallographic studies which indicated that the single stranded helix is favoured by the presence of lipid (Wallace and Janes 1991; Katsaras et al. 1992) while double stranded helices were identified when the crystals were grown under conditions which do not involve lipids (Koeppe et al. 1978; Wallace and Ravikumar 1988; Langs 1988; Langs et al. 1991). Chemical modifications of the peptide may also induce similar effects. The first indication of this phenomenon was provided by the comparative study of GA and GM^-, the latter containing four phenylalanines instead of four tryptophans (Heitz et al. 1982). This substitution leads to a marked lowering of the channel conductance and, when dissolved in chloroform, to a modification of the monomer–dimer equilibrium. The equilibrium constants are $3 \cdot 10^3$ and $3 \cdot 10^4\ M^{-1}$ for GA and GM^- respectively, indicating that the double stranded helix is favoured for the latter peptide (Heitz et al. 1986). Similarly, but to a non-determined extent, substitution of the C-terminal ethanolamine moiety by methylamine, for example (Trudelle et al. 1987), also gives rise, when dissolved in chloroform, to a modification of the monomer–dimer equilibrium toward the dimer.

All these observations raise the following question: do the chemical substitutions which can modify the channel conductance also govern both the conformational and interacting properties of the various gramicidins when incorporated into lipid media? In order to answer this question, at least in part, we tried to relate their conformation(s) with their properties when in a lipid medium, i.e. their conductances and their monolayer behaviour in the same environment, namely glycerylmonooleate (GMO), which will be considered hereafter as a lipid as it mimics the lipidic medium. This was achieved through a combined study involving monolayer and infrared investigations. We will examine here two types of analogues.

Correspondence to: F. Heitz

Chemical structures of the gramicidin A analogues mentioned in this paper. The differences from gramicidin A are underlined.

GM^-: (HCO-DVal[1]-Gly[2]-DAla[3]-Leu[4]-DAla[5]-Val[6]-DVal[7]-Val[8]-DPhe[9]-Leu[10]-DPhe[11]-Leu[12]-DPhe[13]-Leu[14]-DPhe[15]-NHC_2H_4OH)

GT: (HCO-Val[1]-Gly[2]-Ala[3]-DLeu[4]-Ala[5]-DVal[6]-Val[7]-DVal[8]-Tyr[9]-DLeu[10]-Tyr[11]-DLeu[12]-Tyr[13]-DLeu[14]-Tyr[15]-NHC_2H_4OH)

GN: (HCO-Val[1]-Gly[2]-Ala[3]-DLeu[4]-Ala[5]-DVal[6]-Val[7]-DVal[8]-Nal[9]-DLeu[10]-Nal[11]-DLeu[12]-Nal[13]-DLeu[14]-Nal[15]-NHC_2H_4OH)

$GN^{9,15}W^{11,13}$: (HCO-Val[1]-Gly[2]-Ala[3]-DLeu[4]-Ala[5]-DVal[6]-Val[7]-DVal[8]-Nal[9]-DLeu[10]-Trp[11]-DLeu[12]-Trp[13]-DLeu[14]-Nal[15]-NHC_2H_4OH)

$GN^{11,13}W^{9,15}$: (HCO-Val[1]-Gly[2]-Ala[3]-DLeu[4]-Ala[5]-DVal[6]-Val[7]-DVal[8]-Trp[9]-DLeu[10]-Nal[11]-DLeu[12]-Nal[13]-DLeu[14]-Trp[15]-NHC_2H_4OH)

The first one is GT (Trudelle and Heitz 1987); it contains four tyrosines instead of tryptophans and shows conductance properties close to these of GA (Benamer et al. 1993). The second type of analogue is built of a series of naphthylalanine (Nal) containing analogues where the Nal residues vary in number and position (GN: four Nal residues in positions 9, 11, 13 and 15; $GN^{9,15}W^{11,13}$: two Nal residues in 9 and 15 and two Trp in 11 and 13; $GN^{11,13}W^{9,15}$: two Nal residues in 11 and 13 and two Trp in 9 and 15) with conductance properties depending on the number of Trp-Nal substitutions (Heitz et al. 1988; Daumas et al. 1991).

Materials and methods

All samples used in this study were described by Trudelle and Heitz (1987) (GT) and Ranjalahy-Rasoloarijao et al. (1989) (Nal containing analogues) except for GA which was commercial gramicidin D from Sigma (St. Louis, MO). Lipids, dioleoylphosphatidylcholine (DOPC) and glycerylmonooleate (GMO) were also purchased from Sigma.

Infrared spectra were recorded on a Bomem model DA8 spectrometer working in the FT mode and under vacuum. Samples were prepared either by deposition of a drop of a solution of the gramicidin on a CaF_2 plate or by transfer of 4 to 8 monolayers onto CaF_2 plates using the procedure reported by Briggs et al. (1986). The monolayer pressure was maintained constant during the transfer procedure by lowering the surface area of the monolayer through a mobile barrier connected to a home made tensiometer based on the Wilhelmy method. All transfers were achieved at pressures lower than those corresponding to the phase transitions, i.e. below those corresponding to the formation of the close packed monolayers.

Force-area measurements were obtained by spreading a solution of gramicidin/GMO or gramicidin/DOPC (1 to 2 mg/ml in $CHCl_3$ or $CHCl_3/CH_3OH$ mixtures) on a Teflon Langmuir trough with a starting molecular density of $2 \cdot 10^{-3}$ mol $\cdot$ Å^{-2}, and the film was compressed continuously with a Teflon barrier at a compression rate of 5 or 10 Å$^2 \cdot$ mol$^{-1} \cdot$ min^{-1}. The surface pressure was measured using a tensiometer (Prolabo, Paris) based on the Wilhelmy method. The surface tension of the pure water before spreading the materials was 72 mN/m. The results reported here are an average of at least 5 measurements.

Results and data interpretation

Comparison GA–GT

An analysis of the ion transfer properties through lipid bilayers induced by GA and GT indicates that these gramicidins behave very similarly, although the conductance of the synthetic analogue is somewhat lower than that of the natural peptide (67 pS for GT in 1M CsCl, GMO/decane membranes which has to be compared with 82 ps for GA under identical conditions). On the basis of a 3B2S model (see following scheme) in a CsCl medium, this channel conductance lowering on going from GA to GT has been attributed to a decrease of the rate constant corresponding to the binding process (Benamar et al. 1993).

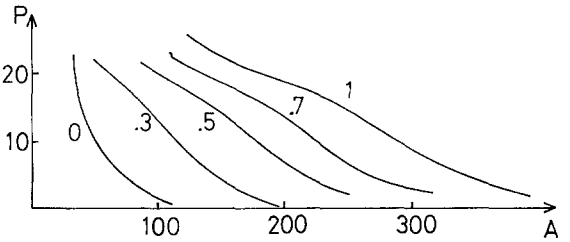


Fig. 1. Variations of the surface pressure P (in mN/m) with the molecular area A (in Å^2) at various GA/GMO ratios in molar fractions as indicated on the figure

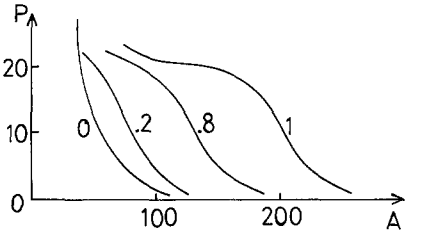
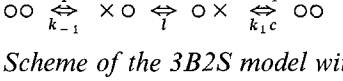


Fig. 2. Variations of the surface pressure P (in mN/m) with the molecular area A (in Å^2) at various GA/GMO ratios in molar fractions as indicated on the figure

Table 1. Molecular area in Å^2 of the gramicidin analogues as determined at the close packed situation of monolayers containing only the peptides

GA	GT	GN	$GN^{9,15}W^{11,13}$	$GN^{11,13}W^{9,15}$
230	190	180	220	185



Scheme of the 3B2S model with one ion in the channel. o *empty site;* × *occupied site.* k_1, k_{-1} *and* l *are the binding, dissociation and translocation rate constants respectively and* c *is the cation activity.*

As to their monolayer behaviour, examination of the force area plots together with the molecular area corresponding to the close packed monolayer would suggest that GA strongly differs from GT. Indeed, beside a lowering of the molecular area on going from GA to GT (see Table 1), the phase transition of GA appears only as a shoulder, while it is well defined in the case of GT (Figs. 1 and 2 and see Fig. 1 in Davion–Van Mau et al. 1987). These differences may be related to the chemical structures of the two gramicidin, more precisely to differences in bulkiness and hydrophobicity and therefore in the mobility of their aromatic side-chains. When studied in the presence of lipid (DOPC or GMO), whatever the peptide/lipid ratio (X), the force area plots always show the same trends, which resemble those obtained for monolayers

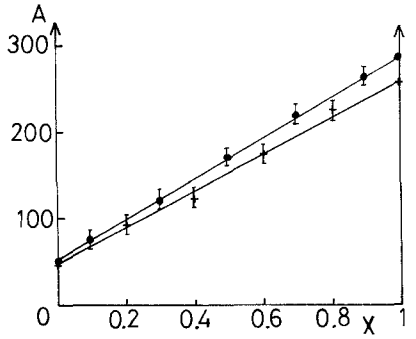


Fig. 3. Variations at 10 mN/m of the mean molecular areas with the peptide/lipid ratios X (in molar fractions) for (o) GA and (+) GT. The bars indicate the statistical errors

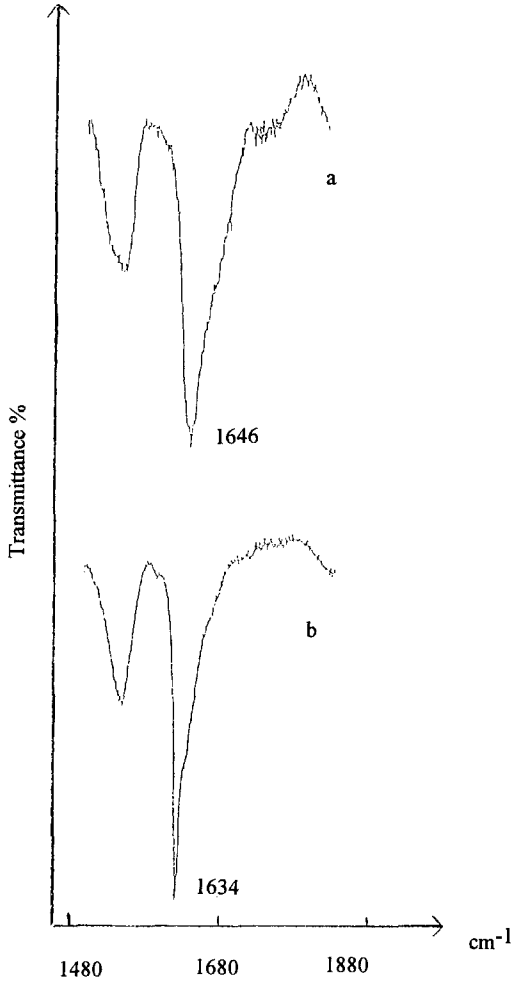


Fig. 4. Infrared spectrum of transferred monolayers of **a** GA at 10 mN/m; $X=1$; **b** GN at 10 mN/m; $X=0.1$. The spectra of the other gramicidins are not shown as they are very similar to these shown in this figure (see text)

containing only the corresponding peptides (see Figs. 1 and 2). In the case of GA, examination of the variation of the mean molecular area with X (Fig. 3), at a given surface pressure below the phase transition, indicates that in a GMO medium the linear behaviour is identical to that described earlier using DOPC as lipid (Van Mau et al. 1988). The same behaviour with a linear variation of the mean molecular area with X is obtained with GT (Fig. 3). This observation together with the fact that, at any X, the phase transition always occurs at the same surface pressure (Fig. 2) indicate that, according to Crisp's rule (Gaines 1965), this analogue, just like GA (Van Mau et al. 1988), is not miscible with the two lipids GMO and DOPC. As to the conformational state of GT, infrared spectra obtained on transferred monolayers built of both the lipids and the peptide indicate that the amide I band lies at $1646 \pm 3\ cm^{-1}$, which is the same as that found for GA (Fig. 4) (Benayad et al. 1991). Such a position for an amide I band was identified by X-ray diffraction studies made on a synthetic poly-D–L-peptide as characteristic of a single stranded Π_{DL} helical structure (Heitz et al. 1975).

It must, however, be noted that in the case of monolayers made of pure peptide, some differences in behaviour between GA and GT may occur. Indeed, while the infrared spectrum of GA in the 1500–1700 cm^{-1} region, always remains identical (amide I band centered at 1646 cm^{-1}), that of GT depends on the chloroform content of the starting solvent mixture (chloroform + methanol). An increase of the chloroform content from 50 to 70% leads to a change of the amide I position, which moves from 1646 to 1633 cm^{-1} and suggests a monomer–dimer transition, as deduced from the wavenumber–conformation relationship established for alternating D–L-peptides. While the 1646 cm^{-1} bands can be attributed to the monomer (see above) the 1633 cm^{-1} band, which usually characterizes a β sheet structure in all L-peptides, is indicative of an antiparallel double helix or dimer in the case of D–L-peptides (Veatch et al. 1974; Lotz et al. 1976).

The present study clearly indicates that, except for the solvent influence described above, both GA and GT have very similar behaviour and, assuming that the transfer procedure does not modify the conformational state of the peptide, that they adopt the single stranded Π_{DL} helical conformation when incorporated in a lipid medium. It is also shown that both gramicidins are not miscible with GMO or DOPC.

Naphthylalanine containing gramicidins

From previous investigations dealing with single channel experiments carried out on GA analogues bearing non polar aromatic side-chains instead of the indoles of the tryptophan residues, it was concluded that:

– replacement of the four Trp residues by non polar aromatic residues such as phenylalanine (GM) or naphthylalanine (GN) leads to a strong lowering of the channel conductance which becomes voltage dependent. Furthermore, this lowering cannot be attributed to an effect induced by differences lying in the bulkiness of the aromatic side-chains as both GM and GN show the same conductances.

– the study of a series of gramicidin analogues where the number and position of the polar residues is varied indicated that the conductance is mainly governed by the

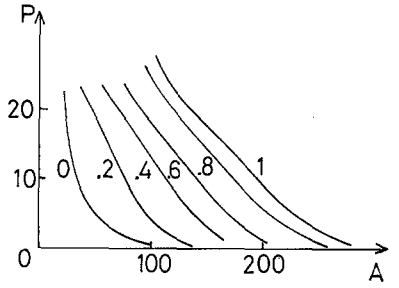


Fig. 5. Variations of the surface pressure P (in mN/m) with the molecular area A (in Å^2) at various $GN^{11,13}W^{9,15}$/GMO ratios in molar fractions as indicated on the figure

Table 2. Phase transition pressures in mN/m of the naphthylalanine containing analogues of gramicidin A as a function of the peptide/lipid ratio X

X	0	0.1	0.2	0.3	0.4	0.5	0.6	0.7	0.8	0.9	1
$GN^{9,15}W^{11,13}$	44	/	18	/	/	18	17.2	/	16.8	17.8	17
$GN^{11,13}W^{9,15}$	43	/	/	16	/	15.6	15.6	16.2	/	/	15.6
GN	45	18,2	16	14	13.2	12.4	10.2	9.4	/	8.9	8

number of polar residues, depends little on their positions, and follows the scale (Daumas et al. 1991)

$$GA > GN^{9,15}W^{11,13} \approx GN^{11,13}W^{9,15} > GN \approx GM$$

Although all three Nal-containing gramicidins, namely GN, $GN^{9,15}W^{11,13}$ and $GN^{11,13}W^{9,15}$, show a linear variation of the mean molecular area with the peptide/lipid ratio (X), they can be divided into two groups depending on the change with X of the phase transition.

The first group is built of GN and it is characterized by a phase transition which, although difficult to determine accurately, clearly depends on X (see Fig. 5 and Table 2). This observation provides a good basis for stating that, according to Crisp's rule (Gaines 1965), GN and GMO are ideally miscible, i.e. GN is miscible with GMO without interactions. Concerning the conformational state of the peptide, as the infrared spectrum of GN containing transferred monolayers shows an amide I band centered at 1633 cm^{-1} (Fig. 4) it can be concluded that under these conditions GN adopts a double helical form (see the above discussion), at least for X ranging between 0.1 and 1. This observation shows the influence of the nature of the aromatic side chains on the relative stability of the various possible conformations that the gramicidin molecule can adopt and emphasizes the role of the polarity of these side chains: polar side chains favour the single stranded helical structure (the channel form) while non polar side chains favour double stranded helices.

The second group of Nal-containing analogues includes $GN^{9,15}W^{11,13}$ and $GN^{11,13}W^{9,15}$. For both these gramicidins, in addition to a linear variation of the mean molecular area with X (Fig. 6) their corresponding phase transition pressures remain constant (Table 2) and this is illustrated in Fig. 7 for $GN^{11,13}W^{9,15}$. As this situation is the same as that of GT, it can be concluded that $GN^{9,15}W^{11,13}$ and $GN^{11,13}W^{9,15}$ are not miscible with

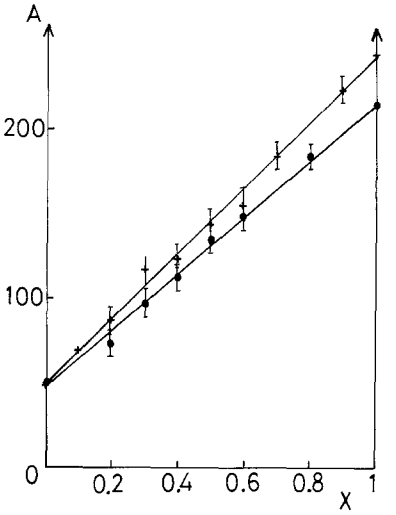


Fig. 6. Variations at 10 mN/m of the mean molecular area with the peptide/lipd ratios X (in molar fractions) for (o) $GN^{11,13}W^{9,15}$ and (+) $GN^{9,15}W^{11,13}$. The bars indicate the statistical errors

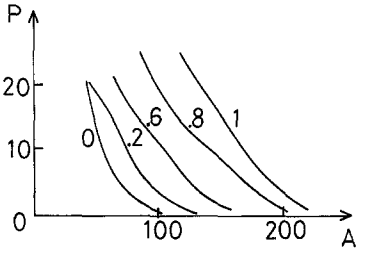


Fig. 7. Variations of the surface pressure P (in mN/m) with the molecular area A (in Å^2) at various GN/GMO) ratios in molar fractions as indicated on the figure

GMO. However, close examination of Fig. 6 and of Table 1 reveals that, although both analogues have the same overall chemical structure, the molecular area of $GN^{11,13}W^{9,15}$ is slightly lower than that of $GN^{9,15}W^{11,13}$, suggesting that the two analogues may adopt different conformations. Infrared measurements made on transferred monolayers reveal that the amide I bands of $GN^{9,15}W^{11,13}$ and $GN^{11,13}W^{9,15}$ lie at 1646 and 1634 cm^{-1} corresponding to single and double stranded helices respectively. Assuming that both gramicidins are aligned at the air–water interface with their helical axis parallel to the interface this observation is consistent with a lower molecular area for the latter. Therefore, the comparison of these two gramicidins suggests that the position of the polar aromatic residues is of major importance with regard to the favoured structure while it barely influences the conductance properties induced by the single stranded forms.

Discussion and conclusion

Let us first examine the consequences of the non miscibility between GA and lipids, especially with regard to the controversy which concerns the smallest conducting unit; dimer (Cifu et al. 1992; Andersen and Koeppe 1992) ver-

sus tetramer (Stark et al. 1986; Stark 1992). First of all it must be recalled that the models proposed by these two groups are deduced from experiments which were carried out under very different conditions. These reported by Stark et al. (1986) deal with macroscopic conductance measurements using highly doped membranes while these of Cifu et al. (1992) were made under single channel conditions. Therefore, owing to the strong tendency for GA to self-associate, together with the fact that this peptide is not miscible with lipids, in highly doped membranes segregation of the gramicidin molecules will occur while this phenomenon will be avoided or strongly reduced under single channel conditions. Hence, it is therefore not surprizing that both aggregates and "isolated" molecules show different behaviour, especially with regard to their kinetics of channel opening. In other words, two aggregates, one on each side of a bilayer will favour the "simultaneous" opening of several channels while only a single opening occurs in the case of isolated species. Thus, in our opinion, the different explanations of the experiments carried out by the two groups derive only from differences in the experimental conditions used for the investigations. The puzzling point that remains is the behaviour of the covalent head to head dimer. Preliminary relaxation experiments carried out on a head to tail 31mer giving rise, in GMO membranes, to single channel events of nearly infinite lifetime (Lelievre et al. 1989) and which do not reveal any relaxation phenomenon favour the above proposed explanation.

Beside the proposal of the above explanation concerning the channel structure, the present investigations indicate that substitutions of Trp residues of gramicidin A by tyrosines, which also bear a dipole moment, do not modify the overall properties of the peptide. This concerns the conductance and the miscibility properties together with the favoured conformational state when in a lipid environment. In contrast, replacement of Trp residues by naphthylalanines can induce drastic modifications of the various properties of the peptide. Although the channel conformation (that which induces the ionophore activity) remains the same, as revealed by the formation of hybrid dimers (Fonseca et al. 1992), the removal of all or part of the polar residues leads to a decrease of the conductance. The amplitude of the decrease is dependent on the number of Trp to Nal substitutions. As to the conformational states of the GA analogues, the present study shows that replacement of polar aromatic residues by non polar residues can favour the double helical form depending on the number and position of the substitutions. An attempt to correlate the favoured conformational state with the ionophore activity failed, mainly due to the fact that $GN^{11,13}W^{9,15}$ and $GN^{9,15}W^{11,13}$ which show nearly identical channel conductances adopt different conformational states. Does this imply that the single stranded helix (the channel form) is of higher energy depending on the number and positions of Trp to non polar substitutions or that the energy of the double stranded helix is lowered while that of the single stranded helix remains equal? Although this question appears important, as it may help to understand the variation of conductance induced by modifications of the aromatic side-chains, we are not able, at present, to decide between these two possibilities. Nevertheless, the experimental results presented here are consistent with the conclusions reported by Hu et al. (1993) who confirmed that the role of the tryptophans lies in a stabilization of the monomeric form due to their favored interfacial situation while these residues are more regularly distributed all along a double helix where some of them (at least Trp 9 and 11) are buried in the hydrophobic medium. A similar role can be attributed to the polar phenol groups of the tyrosine residues. It also appears that, although all Trp residues have similar influence on the channel conductance (Becker et al. 1991), some positions play a particular conformational influence. The present work indicates that, at least, couples 11,13 and 9,15 do not play the same conformational role.

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