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The double ππ5.6 helix of gramicidin A predominates in unsaturated lipid membranes

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Abstract. The structure of the channel-forming polypeptide gramicidin A (GA) incorporated into phosphatidylcholine (PC) liposomes has been studied as a function of the degree of unsaturation of the acyl chains of PC. The initial conformational state of GA in reconstituted bilayers is determined by the solvent in which the peptide and the lipid are initially co-dissolved, whereas the equilibrium conformational state (after heat incubation) is affected by the lipid structure rather than by the nature of the solvent. The conformational equilibrium of GA has been studied in liposomes prepared from PC having a variable number of double bonds in the fatty acid moiety, by circular dichroism and Fourier transform infrared. Liposomes were prepared from trifluoroethanol or ethanol solutions and incubated at 68 °C. GA was shown to retain the conformation of the right-handed $\pi\vec{6}.3\pi\overleftarrow{6}.3$ helix in PC with saturated acyl chains and with one double bond, whereas in dilinoleoyl-PC, having two double bond in each chain, the thermodynamically preferred structures are left-handed antiparallel and parallel double ππ5.6 helices. Natural soybean PC also favours left-handed ππ5.6 helical structures of GA (~75%). This finding is discussed in terms of the role of PC unsaturation in the dynamic properties of the lipid matrix. Differences between observed FTIR spectra of the ↑↓ππ5.6 helix in solution (and to a larger extent in the membrane) and the calculated IR spectra can be interpreted as resulting from deviation of the real structure from the theoretically derived ideal helix. The data obtained provide grounds for better understanding of a GA channel functioning in lipids of variable degrees of unsaturation.

Key words: Gramicidin A – Ion channel – Membrane proteins – FTIR – Circular dichroism

Abbreviations: GA, gramicidin A; CD, circular dichroism; FTIR, Fourier transformed infrared; 2D-NMR, two-dimensional nuclear magnetic resonance; DSPC, distearoylphosphatidylcholine (di-C18:0); DPPC, dipalmitoyl-PC (di-C16:0); DMPC, dimiristoyl-PC (di-C14:0); POPC, palmitoyloleyl-PC (C16:0-C18:1); DOPC, dioleyl-PC (di-C18:1); DLPC, dilinoleoyl-PC (di-C18:2); TFE, trifluorethanol; SDS, sodium dodecylsulfate; TMA-DPH, 1-[4-(trimethylammonio)-phenyl]-6-phenyl-1,3,5-hexatriene; HPLC, high-performance liquid chromatography

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Introduction

Membrane proteins play a key role in ionic transport across biomembranes. Their channel activity is determined by the conformations adopted in the lipid bilayer. Channels may exist in a number of discrete open and closed states. The mechanisms making channels ion specific and allowing them to pass through various gating states remain obscure. In this context the GA channel is an object of intense investigation, since it may represent an interesting system for studying the structure-function relationship of the transmembrane channel. The antibiotic gramicidin A (GA) is a hydrophobic pentadecapeptide produced by *Bacillus brevis*. The GA dimer forms channels (Bamberg and Läuger 1973; Veatch et al. 1975) selective for monovalent cations of alkali metals (Hladky and Haydon 1970, 1972). Studies of GA-induced channel conductance are helpful for understanding the single file ion transport and channel-water-ion interactions and for modelling of ion transport through biomembranes (Fornili et al. 1984; Khutorsky 1984; Benndorf 1989).

In organic solvents GA forms four dimeric species that are in a state of slow equilibrium. For these species Veatch et al. (1974) proposed conformations of parallel and antiparallel double helices (↑↑ππ and ↑↓ππ). It was shown by 2D-NMR in ethanol (Arseniev et al. 1984, 1985a; Barsukov IL et al. 1987a) that species 1 and 2 are left-handed ↑↑ππ5.6 helices (5.6 residues/turn) differing in a relative shift of the polypeptide chains, species 3 is the left-handed ↑↓ππ5.6 helix (the dominant structure in solution determined also by calculation of IR spectra (Sychev et al. 1980)), and species 4 is the right-handed ↑↑ππ5.6 helix.

To obtain the structure of the ion conducting form of GA, many theoretical and experimental studies have been carried out. CD spectra of GA in liposomes of DM-

PC, DPPC, and egg PC have the same shape (in the equilibrium state) and do not depend on the presence of ions (Wallace 1986; Masotti et al. 1980; Sychev and Ivanov 1984). It was also found that the "membrane" curve cannot be described by a linear combination of the CD curves of the species in solution. The GA structure in SDS micelles mimics that in liposomes (CD curves are very similar). The GA structure in detergent micelles was obtained by 2D-NMR (Arseniev et al. 1985b). In SDS, GA was shown to be a dimer of two right-handed π6.3 helices joined in N-terminal-to-N-terminal fashion $\overrightarrow{\pi 6.3}\,\overleftarrow{\pi 6.3}$ and thus differing from the structure proposed by Urry et al. (1971a, b) in having the opposite helical sense. Thus, current views strongly favour the right-handed $\overrightarrow{\pi 6.3}\,\overleftarrow{\pi 6.3}$ helix as the only ion conducting species in biological and artificial membranes.

At the same time, two different structures were found for GA bis-derivatives in solution (Ivanov and Sychev 1982) and two populations of channels were formed by these compounds in bilayer lipid membrane (Irkhin et al. 1984), indicating the possibility of double helical channel formation. Recent investigations by Rottenberg and Koeppe II (1989) provided data favouring formation of "non-standard" channels formed by desformyl GA and des(formyl-valyl) GA, which may contribute to the conductance. However, the conductivity data obtained could not be unequivocally interpreted without parallel structural investigations of the polypeptide in the same lipid membranes. Because of the structural polymorphism of GA, the channel structure may depend on the membrane composition, and that is why a conformational study of GA in membranes made of various lipids seemed necessary. Killian et al. (1988) showed that an increase of the fatty acid chain length in saturated PC (C12:0, C14:0, C16:0), and also in C18:1 and C22:1 does not significantly change the CD spectra of GA in liposomes, indicating that the $\overrightarrow{\pi 6.3}\,\overleftarrow{\pi 6.3}$ helix remains the preferred conformation in these mebranes.

The present paper compares GA structures in membranes of saturated (DPPC, DMPC, DSPC) and a set of unsaturated (POPC, DOPC, DLPC) lipids. Lipids with unsaturated fatty acids play an important role in the lipid matrix structure and dynamics, which in turn influence the lipid-protein interaction and the secondary structure of membrane proteins, as well as their activity (Lenaz and Castelli 1985). In this respect the structural changes of a channel-former resulting from changes in the fatty acid composition of phospholipids may be of interest not only for GA functioning.

It is known that the structure originally formed by GA in a membrane depends on the solvent used to prepare the liposome dispersion (Masotty et al. 1980; Killian et al. 1988; Lograsso et al. 1988). For example, when TFE is used for co-solubilization of GA/DMPC the $\overrightarrow{\pi 6.3}\,\overleftarrow{\pi 6.3}$ helix is formed, while with ethanol, the measured CD spectrum corresponds neither to the $\overrightarrow{\pi 6.3}\,\overleftarrow{\pi 6.3}$ helix, nor to double helices. However, long-term incubation of the sample (>8 h, 68 °C) after reconstitution of GA in liposomes of saturated lipids transforms the polypeptide structure into the $\overrightarrow{\pi 6.3}\,\overleftarrow{\pi 6.3}$ helix, which is the thermodynamically preferred one for these lipids (Masotty et al. 1980; Killian et al. 1988). To determine the equilibrium GA conformation in different unsaturated lipids, we used a similar approach. Liposomes containing GA were prepared from ethanol or TFE, then we followed the CD spectra change in the course of incubation (68° C). During that time the sample reaches thermodynamic equilibrium. For identification of the polypeptide structure we also used FTIR spectroscopy which provides additional valuable information.

Materials and methods

Materials

Gramicidin as a mixture of gramicidin A (~85%), B and C was obtained from Boehringer. DPPC, DSPC, DMPC, POPC, and DLPC were from Serva. DOPC and soybean PC were from Sigma; TFE and SDS were from Fluka. Chloroform solution of DLPC was supplied and stored in sealed glass ampoules. Lipids were stored at −20 °C and their purity was checked by TLC.

Preparation of liposomes and micelles

Liposomes were prepared according to Killian et al. (1988). Stock solutions of the appropriate amount of GA (0.20–2.6 mg) and lipids were prepared in methanol (0.8 ml) and chloroform (0.8 ml), respectively. The solutions were mixed and solvents were removed on a rotary evaporator. The dry mixture was then dissolved in the organic solvent of choice (1.6 ml). After incubation for 2 h at room temperature the solvent was removed by rotary evaporator (at 45 °C) and samples were dried for 2 h under high vacuum (~10^{-3} Torr). The mixtures were hydrated in 10 mM NaCl in H_2O (pH 6.5) to a GA concentration of $2-5\times10^{-4}$ M for CD and $4-5\times10^{-3}$ for FTIR measurements. The lipids were allowed to swell for 30 min at room temperature or in the case of DPPC and DSPC at 45 °C and 68 °C respectively. Then, the samples were sonicated (except for ^{31}P-NMR measurements) at 20 °C (or at 45 °C and 68 °C in the case of DPPC and DSPC respectively) by a B. Braun Melsungen AG sonicator (Braun-sonic 1510) for 2–5 min until they become optically clear. Heat incubation of the samples was done after purging and filling of flasks with argon. The extent of oxidation of the unsaturated lipids during heat incubation was controlled by the D_{233nm}/D_{215nm} ratio (Klein 1970) for the samples without GA. For more unsaturated DLPC and soybean PC the oxidation is not significant during incubation for 3–5 h (respectively), but it is notably accelerated upon more prolonged incubation (oxidation index raised to ~0.4). This results in deterioration of the signal/noise ratio in the CD spectra (200–230 nm) of the corresponding samples with GA.

When studying GA in the presence of salts, the GA-cation complex in the chloroform-methanol (1:1) solution was produced by adding the salt solution (0.15 M) in methanol to the salt-free GA solution (10^{-4} M), followed by the addition of the appropriate volume of chloroform

(to keep the 1:1 ratio of methanol/chloroform). The sample was equilibrated for 1 day. Then liposomes were prepared as usual, except that 2 M salt solution in H_2O was used.

All presented spectra were measured at a peptide/lipid molar ratio of 1:25–1:30 (except for DPPC where a 1:150 ratio was also used). Ratios were derived from determinations of the lipid concentration by the phosphorus assay (Gerlach and Deuticke 1963) and GA concentration by measurements of absorbance at 280 nm after 50-fold dilution of the sample in methanol (except for DLPC and soybean PC, where the GA concentration was calculated from its initial mass).

Incorporation of GA into SDS micelles was carried out as described by Arseniev et al. (1985). The concentration of GA was 5.3×10^{-3} M, and the SDS/GA molar ratio was 50, this corresponds to incorporation on average of not more than two GA molecules in one micelle. Size measurements of the obtained GA/SDS micelles, made using photon correlation spectroscopy, showed that tetramers or higher aggregates do not form and therefore that every micelle contains a GA dimer (Sychev and Barsukov IL, unpublished).

Circular dichroism

Spectra were recorded at 20 °C on a Jasco 500 C spectropolarimeter in demountable cells (Hellma) with 10^{-2} and 10^{-3} cm optical path length. The spectra reported are the average of 2–3 scans. The spectrum of the appropriate suspension of liposomes without GA was used for the baseline. The curves were analyzed using a least-squares fitting procedure at 2 nm intervals in the 200 to 250 nm region (the shorter wavelength region was not included because of possible distortion of CD curves by light-scattering effects).

^{31}P-Nuclear magnetic resonance

81 MHz ^{31}P-NMR spectra were recorded on a Bruker MSL-200 NMR spectrometer at 20 °C (27.3 mg of lipid in 2 ml volume).

Fluorescence

Spectra were recorded at 20 °C on a Hitachi MPF4 spectrofluorimeter with a MPF Data Processor after 100-fold dilution of the sample.

Fourier transformed infrared spectroscopy

Measurements were made at 20 °C on a Perkin-Elmer 1725 X FTIR spectrometer with a hermetic interferometer area previously purged by dry nitrogen to eliminate water vapour absorption. The cell thickness was 100 μm for dioxane solution. Spectra of water suspensions were measured in a home-made demountable CaF_2 cell with 12 μm path length. The cell thickness was determined by the interference technique. 200 and 500 scans at 4 cm^{-1} resolution were coadded for dioxane solution and water suspension, respectively. In the later case a difference spectrum was generated by subtraction of an "empty" liposomes (or SDS micelles) spectrum from the spectrum of GA containing liposomes (or micelles). The difference factor was varied in the 0.99 to 1.14 region to yield a flat baseline at 1450–1800 cm^{-1} and an amide II/amide I ratio equal to one in the spectra of GA in organic solvents. A smoothing level of 3 was applied.[1]

Results

Conformational changes of GA determined by CD spectroscopy

Let us first consider the CD spectra of GA in lipids, beginning with saturated DPPC and unsaturated POPC. Spectra of GA in these lipids, when using TFE (Figs. 1 A and 2 A), are similar to that of GA in SDS micelles, especially at a peptide/lipid molar ratio 1:150, when the equilibrium GA structure is readily obtained (Sychev and Ivanov 1984). According to NMR data (Arseniev et al. 1985 b) this curve corresponds to a right-handed $\overrightarrow{\pi 6.3}$ $\overleftarrow{\pi 6.3}$ helix, which forms the ion channel. Upon incubation the spectra change slightly (Figs. 1 A and 2 A). Initial use of ethanol solution leads originally to notably different curves with a strong negative band at 229 nm. However, upon incubation the spectra reproduce those obtained with the use of TFE (Figs. 1 B and 2 B).

The spectrum of GA in the saturated lipid, DSPC (Fig. 3) has a weaker intensity for the peak at 218 nm in comparison with DPPC, DMPC (not shown, see Killian et al. (1988)) and POPC. When using TFE the spectrum of GA in DSPC, as well as in the above mentioned lipids, changes slightly upon incubation. In the case of ethanol the spectrum is shifted again toward the CD curve of GA in SDS micelles (cf. Figs. 1–3).

Opposite spectral changes are observed in liposomes made of DLPC (Fig. 4). The "channel" type curve with a positive 218 nm band obtained with TFE transforms upon heat incubation to the curve with two strong negative bands at 210 and 229 nm. Incubation of GA in DLPC liposomes prepared from TFE and ethanol for more than 3 h does not result in significant changes of the curve (Fig. 4 A, curves *c* and *d*[1]). Thus, the spectra obtained correspond closely to the equilibrium state of GA in this lipid. At the same time, the difference observed between the 3 h-curves in Fig. 4 A, B indicates that the new GA state is best expressed with the ethanol + incubation schedule (see also below Fig. 6). In doing so

[1] However, when the incubation time is >4 h the ratio D_{233nm}/D_{215nm} increases (see Methods) and the signal/noise ratio in the CD spectra decreases, implying that the lipid is oxidised. Therefore curve *d* was recorded with less accuracy than the other curves. For the same reason, the spectrum of GA in DLPC from ethanol was not measured accurately (in the 200–230 nm region) after incubation for more than 3 h (spectrum not shown)

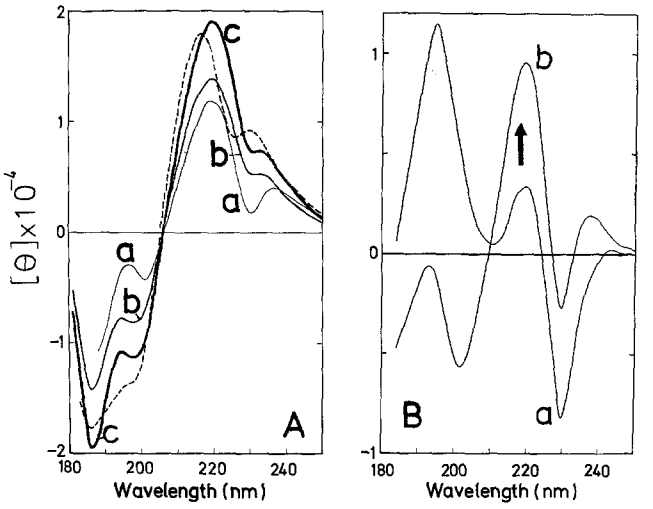

Fig. 1. CD spectra of GA in DPPC liposomes prepared from TFE **A** and ethanol **B:** *a* before incubation, *b* after incubation at 68 °C for 8 h **A** and 10 h **B**. Curve *c* in panel A – the same as *b* but obtained at peptide/lipid molar ratio 1:150 instead of 1:30. Spectrum of GA in SDS micelles is shown by a dotted line **A** for comparison. The direction of the curve shift during incubation is shown by arrows on this figure and on the following ones

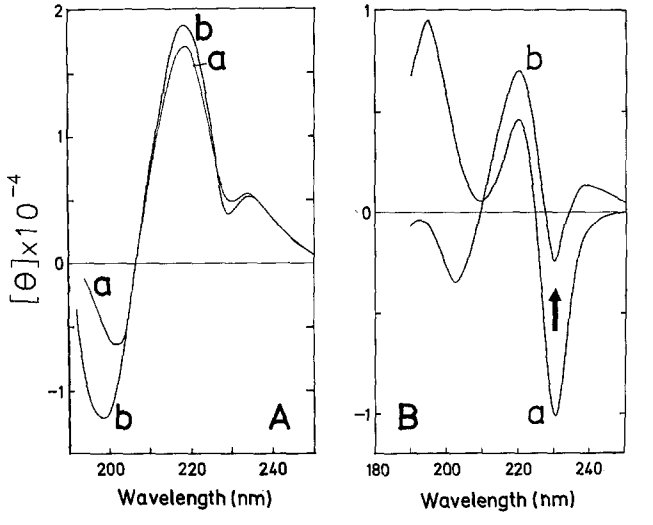

Fig. 2. CD spectra of GA in POPC liposomes prepared from TFE **A** and ethanol **B:** *a* before and *b* after incubation at 68 °C for 8 h

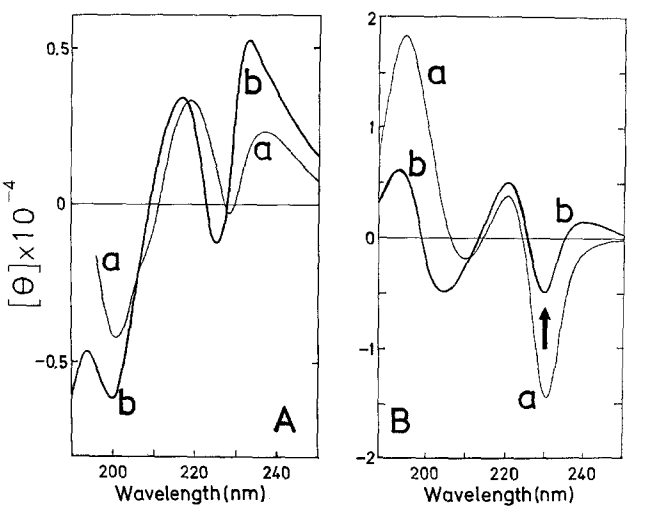

Fig. 3. CD spectra of GA in DSPC liposomes prepared from TFE **A** and ethanol **B:** *a* before and *b* after incubation at 68 °C for 8 h

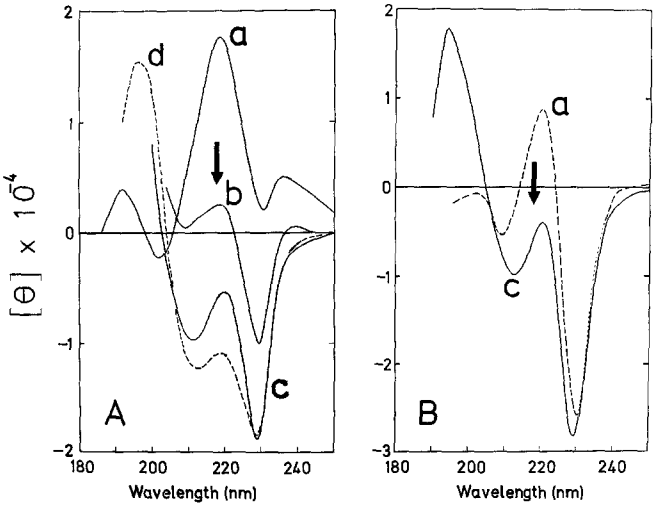

Fig. 4. CD spectra of GA in DLPC liposomes prepared from TFE **A** and ethanol **B** with different periods of heat incubation at 68 °C: *a* 0 h, *b* 1 h, *c* 3 h, *d* 4 h

the conformational rearrangement in unsaturated lipids is faster than in saturated ones, where the equilibrium state is reached upon incubation for >8 h (Fig. 1 B; Killian et al. (1988)).

The CD spectra in DPPC and POPC, as well as their changes upon incubation, are similar to those previously observed for DMPC and DPPC (Wallace 1986; Killian et al. 1988; Sychev and Ivanov 1984). However, the spectral changes and the final curve of GA in unsaturated DLPC are new. In other words, the initial conformational state of the polypeptide in the membrane after reconstitution is determined by the solvent used for liposome preparation. The final conformational equilibrium (after heat incubation of the sample) is, in contrast, affected by the lipid nature, rather than the solvent. Moreover, the equilibrium state of GA in unsaturated lipids has CD spectra differing from those in saturated lipids or in POPC.

Figure 5 shows spectra of GA (after heat relaxation) in liposomes obtained from TFE. The spectral shape changes monotonically with increasing unsaturation of the PC lipids. Besides, the presence of one double bond in one or both acyl chains of POPC and DOPC does not promote such a major change in channel-former structure as does the presence of two double bonds in DLPC. It is of interest that the structure of GA in the natural mixture of soybean PC containing unsaturated lipids with 2 and 3 double bonds is similar to the structure in DLPC. The rates of the conformational transition in these lipids are also close to each other (cf. Figs. 4 and 6).

To confirm the bilayer organization of the lipid after prolonged heat incubation of GA/unsaturated lipid the ^{31}P-NMR spectrum (not shown) of the dispersion GA/soybean PC (from ethanol) was recorded. The spectrum shows the "bilayer" type ^{31}P-NMR signal (de Kruijff et

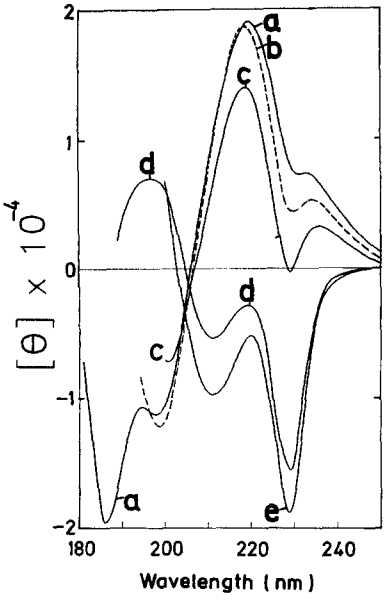

Fig. 5. CD spectra of GA in liposomes made of different lipids, prepared from TFE after incubation at 68 °C: *a* DPPC, 8 h, *b* POPC 8 h, *c* DOPC, 8 h, *d* soybean PC, 5 h, *e* DLPC, 3 h

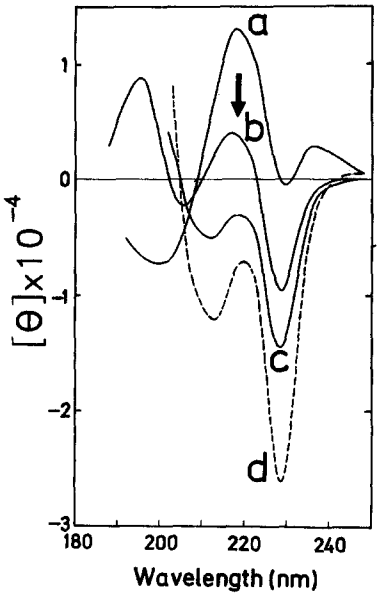

Fig. 6. CD spectra of GA in soybean PC liposomes prepared from TFE with different periods for heat incubation at 68 °C: *a* 0 h, *b* 3 h, *c* 5 h, and prepared from ethanol with incubation period 5 h *d*. Similar results were obtained when unsonicated samples (prepared for ^{31}P-NMR) were used

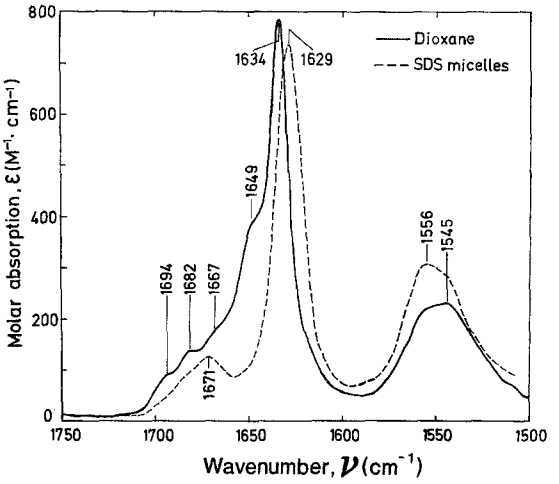

Fig. 7. FTIR spectra in amide I and II region of GA in dioxane solution (—) and in SDS micelles (– – –). Concentration of GA, 1.0×10^{-3} M and 5.3×10^{-3} M respectively

al. 1988; Tournois et al. 1987) and no H_{II} of the ^{31}P-NMR line shape could be detected.

Incorporation of GA into the bilayer was monitored by tryptophan fluorescence spectra. The position of the emission maximum before (331–332 nm) and after (330–331 nm) incubation of the samples indicates that all the tryptophan residues are in a hydrophobic environment, i.e. GA in both cases is incorporated into the hydrophobic part of the membrane. The observed changes of the CD spectra therefore result from structural changes of GA in the bilayer rather than from a polarity change of the environment.

Figure 1 A shows a weak dependence of the GA spectra in DPPC liposomes on peptide/lipid molar ratio. For the other lipids used we observed practically the same CD spectra, independent of the peptide/lipid molar ratio in the range 1:20 to 1:30. Therefore, in these ratio limits, only minor changes of CD spectra were observed, in contrast to the major changes due to the shift from the saturated lipids to the unsaturated ones.

Strong changes in CD spectra upon transition from saturated to unsaturated lipids include the range of tryptophan chiroptical effects at 228 nm, as well as the peptide $n \rightarrow \pi^*$ (218 nm) and $\pi \rightarrow \pi^*$ transitions. It is seen from Figs. 1–6 that these changes are interrelated i.e. changes in the former range are always accompanied by changes in the latter. Therefore it is most likely that the changes reflect major conformational transitions in the peptide backbone, rather than in side chains only. Negative CD curves of GA in the 200–250 nm range have been assigned to left-handed, and positive curves – to the right-handed helices (Sychev et al. 1980). This assignment has been confirmed by the 2D-NMR technique (Arseniev et al. 1984, 1985a, b). Thus, we suggest that GA in unsaturated lipids also forms left-handed helices. For more specific structural assignments we used FTIR spectroscopy.

Single stranded and double stranded helices from FTIR data

FTIR spectra of GA in the model systems are presented in Fig. 7. According to 2 D-NMR data (Arseniev et al. 1984, 1985b), GA in SDS micelles forms a $\overrightarrow{\pi 6.3}\,\overleftarrow{\pi 6.3}$ helix and in dioxane (species 3) a $\uparrow\downarrow \pi\pi 5.6$ helix, which allows for appropriate IR spectral assignment. The infrared spectrum of the $\uparrow\uparrow \pi\pi 5.6$ helix differs only slightly from that of the $\uparrow\downarrow \pi\pi 5.6$ helix (spectra taken by Veatch et al. (1974); structure assignment by Barsukov IL et al.

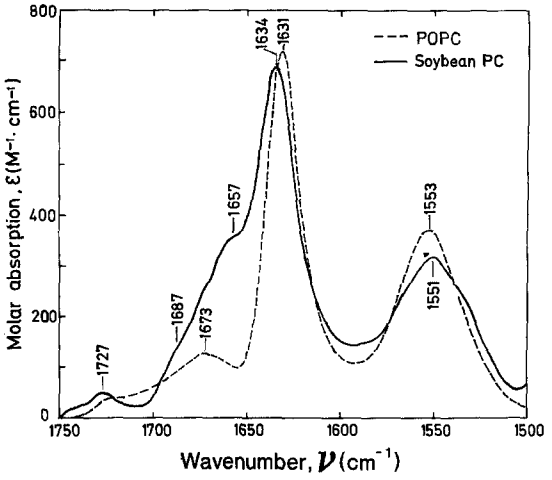

Fig. 8. FTIR spectra in amide I and II region of GA in liposomes of POPC (from TFE) (– – –) and soybean PC (from ethanol) (—) after incubation for 5 h. (Corresponding CD curves see in Figs. 2 and 6.) Concentration of GA, $(4-5)\times10^{-3}$ M. Weak bands at 1720–1730 cm^{-1} are due to incomplete compensation of the phospholipid absorption, $\nu(C=0)$

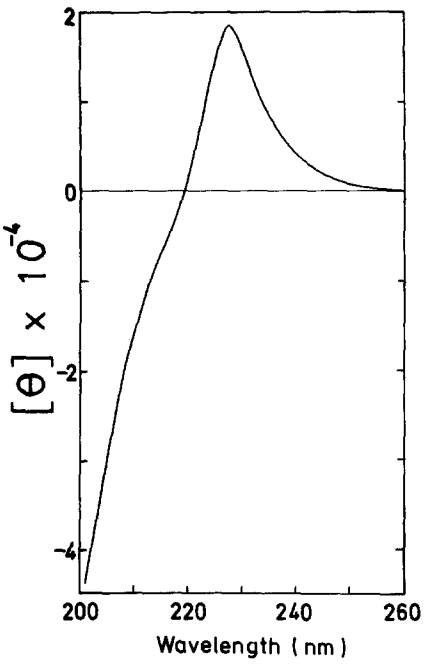

Fig. 9. CD spectra of GA–Cs^+ complex in chloroform/methanol (1:1) solution. Concentration of GA, 2.2×10^{-2} M (dimeric state of GA). The molar ratio $[Cs^+]/[GA]=1.3$

(1987a)). Characteristic features of the spectra of both parallel and antiparallel ππ5.6 helices are a maximum frequency at 1634 cm^{-1}, the 1649 cm^{-1} shoulder at 0.5 peak height (dioxane, ethanol) and week components at 1670–1700 cm^{-1} (Figs. 7 and 12, see below).

Figure 8 presents spectra of GA in POPC (from TFE) and in soybean PC (from ethanol), i.e. in the polypeptide states with opposite CD curves (Figs. 2A and 6). The former spectrum is close to that of GA in SDS and corresponds to the $\overrightarrow{\pi 6.3}\,\overleftarrow{\pi 6.3}$ helix, which agrees with CD data. On the other hand, the FTIR spectrum of GA in soybean PC is similar to that in dioxane, indicating formation of the ππ5.6 helix. The spectrum typical of ππ5.6 helices is also observed for GA in unsaturated DLPC (not shown). In the case of saturated DPPC and DMPC, as we showed earlier (Sychev and Ivanov 1982), the shoulder at 1650 cm^{-1} is absent and the maximum frequency is 1629–1631 cm^{-1}, which corresponds to the $\overrightarrow{\pi 6.3}\,\overleftarrow{\pi 6.3}$ helix. Summarizing, the FTIR data indicate that the predominant GA conformations in the membranes of unsaturated lipids are ππ5.6 helices, in contrast to saturated lipids where the $\overrightarrow{\pi 6.3}\,\overleftarrow{\pi 6.3}$ helix is formed.

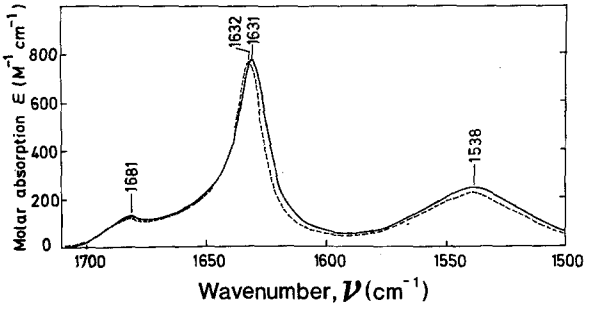

Fig. 10. Infrared spectra of GA–Cs^+ complex in chloroform/methanol (1:1) (—) and in ethanol solution (– – –). Molar ratio $[Cs^+]/[GA]=3$. Concentration of, GA 1.1×10^{-2} M (ensuring complete dimerization)

ππ5.6 and ππ7.2 helices from the CD and FTIR data

Arseniev et al. (1985c) showed by 2D-NMR that the GA-caesium complex in chloroform/methanol solution is a right-handed ↑↓ππ7.2 helix. Figures 9 and 10 present the infrared and CD spectra of the complex obtained under the same conditions and also its infrared spectrum in ethanol. The CD spectrum differs from that of the ππ5.6 helix, as well as the $\overrightarrow{\pi 6.3}\,\overleftarrow{\pi 6.3}$ helices.

The infrared spectrum of the ↑↓ππ7.2 helix (Fig. 10), as well as the spectrum of the single stranded helix, has no shoulder at 1650 cm^{-1}, the second component being at higher frequences (1681 cm^{-1}) than in the spectrum of the $\overrightarrow{\pi 6.3}\,\overleftarrow{\pi 6.3}$ helix (1671 cm^{-1}, Fig. 7). Thus, the ↑↓ππ7.2 helix can be identified by CD and FTIR techniques and the results obtained contain no indication of formation of the ↑↓ππ7.2 helix in unsaturated lipids.

↑↑ππ5.6 or ↑↓ππ5.6 helix?

These structures could not be distinguished by their FTIR spectra. In CD spectra of GA in unsaturated lipids (Figs. 4B, 6) the intensity of the 228 nm peak (-2.6–2.8×10^{-4} deg. cm^{-2} $dmol^{-1}$) is very close to the ellipticity of this peak (-2.7–2.9×10^{-4} deg. cm^{-2} $dmol^{-1}$) in the spectra of left-handed ↑↑ππ5.6 and ↑↓ππ5.6 helices (species 1–3) in dioxane solution (Veatch et al. 1974). CD spectra obtained in lipids can be presented as a linear combination of the spectra of a right-handed $\overrightarrow{\pi 6.3}\,\overleftarrow{\pi 6.3}$ helix and left-handed ↑↑ππ5.6 and ↑↓ππ5.6 helices in dioxane solution. As the spectrum of GA in DPPC liposomes (curve *c*, Fig. 1) closely resembles the spectrum of

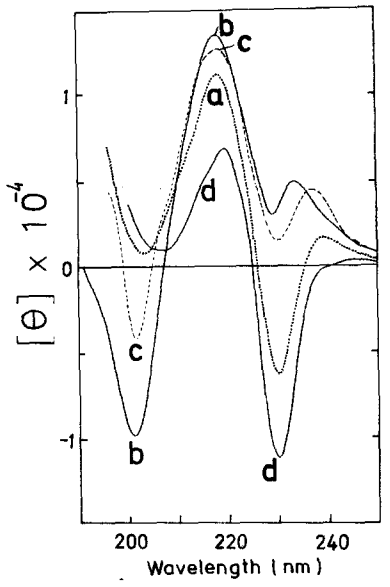

Fig. 11. CD spectra of GA in soybean PC liposomes prepared from chloroform/methanol (1 : 1) in the absence of salts *a*, at 5-fold excess in solution of Cs^+ *b* and K^+ *c* and 10-fold excess of Na^+ *d*

GA in SDS micelles, we assign it to the $\overrightarrow{\pi 6.3}\,\overleftarrow{\pi 6.3}$ helix. These calculations (Table 1) allow us to conclude that right-handed $\uparrow\uparrow\pi\pi 5.6$ helix (species 4) is not present in the equilibrium. Slight changes in CD spectra (cf. Fig. 4 B, curve *c* and Fig. 6, curve *d*) can reflect an important change in the $\uparrow\downarrow\pi\pi 5.6$ vs $\uparrow\uparrow\pi\pi 5.6$ ratio (Table 1), this is a consequence of the similarity of their circular dichroism curves in the 215–250 nm range. In summary, the content of $\uparrow\downarrow\pi\pi 5.6$ and $\uparrow\uparrow\pi\pi 5.6$ helices is similar in unsaturated lipids as well as in solution. However, it is worth noting that the $\uparrow\downarrow\pi\pi 5.6$ vs $\uparrow\uparrow\pi\pi 5.6$ ratio can depend on the lipid structure: in a DLPC-GA bilayer formed from ethanol the $\uparrow\downarrow\pi\pi 5.6$ helix predominates, while in soybean PC under the same conditions the $\uparrow\uparrow\pi\pi 5.6$ helix is predominant.

Effect of Cs^+, K^+ and Na^+ ions on GA structure in liposomes

Before the incorporation of GA into liposomes it was equilibrated in chloroform/methanol + salt solution. Arseniev et al. (1985c) found that under such conditions the GA-Cs^+ complex is formed. Figure 11 presents the CD spectra of GA after its incorporation into liposomes of soybean PC in the presence of a large salt excess. Comparison of these curves with the spectrum of the salt-free state allows us to conclude that Cs^+ and K^+ ions shift the equilibrium towards the $\overrightarrow{\pi 6.3}\,\overleftarrow{\pi 6.3}$ helix, whereas in the presence of Na^+ the equilibrium shifts to left-handed $\pi\pi 5.6$ helices. The last change takes place at a 10-fold (not 5-fold) salt excess, whereas stabilization of the $\overrightarrow{\pi 6.3}\,\overleftarrow{\pi 6.3}$ helix by Cs^+ and K^+ cations occurs at a 5-fold excess. This accords with the conclusion of Hinton et al. (1988) that Na^+ has a significantly lower affinity for GA in lipid than do K^+ or Cs^+.

Table 1. Conformational states of GA in membranes with different lipid composition as calculated from CD data

Lipid	$\overrightarrow{\pi 6.3}\,\overleftarrow{\pi 6.3}$ right-handed	$\uparrow\uparrow\pi\pi 5.6$ left-handed (species 1 + 2)[a]	$\uparrow\downarrow\pi\pi 5.6$ left-handed (species 3)[a]
DPPC (TFE)	1	0	0
DMPC (TFE)	0.85	0.06	0.09
DSPC (TFE)	0.66	0.24	0.10
POPC (TFE)	0.95	0.03	0.02
DOPC (TFE)	0.82	0.09	0.09
Soybean PC (TFE)	0.35	0.32	0.33
Soybean PC (EtOH)	0.24	0.42	0.34
DLPC (TFE, 3 h)	0.29	0.47	0.24
DLPC (TFE, 4 h)	0.20	0.54	0.26
DLPC (EtOH)	0.18	0.33	0.49

[a] CD spectra were taken from Veatch et al. (1974)

Discussion

It follows from the Results section that the predominant conformation of GA in equilibrated membranes of saturated lipids is the right-handed $\overrightarrow{\pi 6.3}\,\overleftarrow{\pi 6.3}$ helix (66–100%, Table 1). The spectrum of GA in saturated DSPC is approximated less satisfactorily by a linear combination of the reference spectra. Special studies are required to find out the reason of some destabilization of the $\overrightarrow{\pi 6.3}\,\overleftarrow{\pi 6.3}$ dimer in the thick DSPC membranes. Probably this fact is related to the mismatch of the bilayer thickness and the length of $\overrightarrow{\pi 6.3}\,\overleftarrow{\pi 6.3}$ helix, as was suggested by Killian et al. (1988). The predominant conformations of GA in equilibrated membranes of unsaturated lipids are left-handed $\pi\pi 5.6$ helices. It is of interest that the natural soybean PC supports predominantly (~75%) the $\pi\pi 5.6$ helical transmembrane structures.

As can be seen from Table 1 the conformational equilibrium at 20 °C does not depend significantly on the variation of the phase state of the lipids (the phase transition temperature, T_m, is equal to 55 °C, 41 °C, 23 °C, −2.6 °C and −21 °C for DSPC, DPPC, DMPC, POPC and DOPC, respectively, Lenaz and Castelli (1985)). As far as the unsaturated lipids are concerned we made measurements at $T > T_m$ and so the observed conformational shift is not due to the effect of temperature. What are the forces responsible for the conformational shift accompanying the increase of lipid unsaturation? Since under these conditions the equilibrium reaches a state similar to that characteristic for solution ($\uparrow\uparrow\pi\pi 5.6 + \uparrow\downarrow\pi\pi 5.6$ helices) we assume that the dynamic properties of the membrane, i.e. its fluidity are the major factors influencing the process. Increases of fluidity brought about by unsaturated lipids, as well as its effect on the structure of membrane proteins, is a widely discussed phenomenon (see e.g. Lenaz and Castelli 1985; Brenner 1984). Van Langen et al. (1989) showed that an increase in lipid unsaturation results in an increase in rotational diffusion coefficient ($D_\perp$) of a fluorescent probe in vesicles and a decrease in the order parameter $\langle P_2 \rangle$. Correlation between the dynamic parameter $D_\perp$ and the fraction of double helices in lipids (Table 2) indirectly supports the membrane fluidity concept discussed above.

Table 2. Fraction of ↑↑ and ↑↓ double helices and diffusion coefficient $D_\perp$ of a fluorescent probe (TMA-DPH) in membranes of different lipids

Lipid	ππ5.6 fraction	$D_\perp$ (ns^{-1})[a]
POPC (TFE)	0.05	0.09
DOPC (TFE)	0.18	0.16
DLPC (EtOH)	0.82	0.27

[a] From van Langen et al. (1989)

Table 3. Frequences (cm^{-1}) of the amide I band for some helical conformations of GA

Structure	Calculated		Observed
	Sychev et al. (1980)	Naik, Krimm (1986)	
↑↓ππ5.6	1634[a]	1636	1634+1649−57 sh
↑↓ππ7.2	1638, 1683	1632, 1677	1631–32, 1681
π4.4 π4.4	1647	1631	1645–48[b]
monomer π4.4	1654	–	1650[c], 1648[d]

[a] The three weak components are not shown (see Figs. 6 and 11)
[b] From Lotz et al. (1976)
[c] From Sychev et al. (1980)
[d] From Heitz et al. (1986)

The present work provides a successful example of the combined application of FTIR and CD techniques for studying the channel-former structures in membranes of variable lipid composition. The first attempt to apply calculation of the amide I band fine structure in studying GA was made by Sychev et al. (1980) when GA was ascribed the ↑↓ππ5.6 helical structure in dioxane solution. Later this structure was confirmed by 2D-NMR (Arseniev et al. 1984). As for the ↑↑ππ5.6 and $\overrightarrow{\pi 6.3}\,\overleftarrow{\pi 6.3}$ helices, also considered in that work, the 2D-NMR data revealed the inaccuracy of calculations based on the atomic coordinates obtained by theoretical conformational analysis of the helices (Collonna-Cesari et al. 1977). Recent studies by 2D-NMR and X-ray confirmed the coordinates obtained theoretically for ↑↓ππ5.6 and ↑↓ππ7.2 (Arseniev et al. 1984, 1985b; Langs 1988), whereas major differences were observed between theoretically deduced and experimentally obtained φ_L, Ψ_L, φ_D, Ψ_D values for ↑↑ππ and $\overrightarrow{\pi 6.3}\,\overleftarrow{\pi 6.3}$ (Arseniev et al. 1985a; Barsukov IL et al. 1987b). Apparently, this is the main reason why computed frequences and resonance splittings of the amide I band for ↑↑ππ5.6 and $\overrightarrow{\pi 6.3}\,\overleftarrow{\pi 6.3}$ helices predicted in Sychev et al. (1980) and Naik and Krimm (1986) are inaccurate, whereas spectra calculated for ↑↓ππ helices correlate well with the experimental data (see below and Fig. 12). Calculations, however, correctly predict lesser splitting in the spectrum of the $\overrightarrow{\pi 6.3}\,\overleftarrow{\pi 6.3}$ helix in comparison with that for ↑↓ππ 7.2 helix. For the latter in the computed and observed (Fig. 10) spectra the shoulder 1650 cm^{-1} is absent and splitting in calculated (Sychev et al. 1980) and observed spectra are very close (Table 3). Experimental and calculated frequences for the π4.4 helix characterized by electron diffraction (Lotz et al. 1976) are also presented in Table 3.

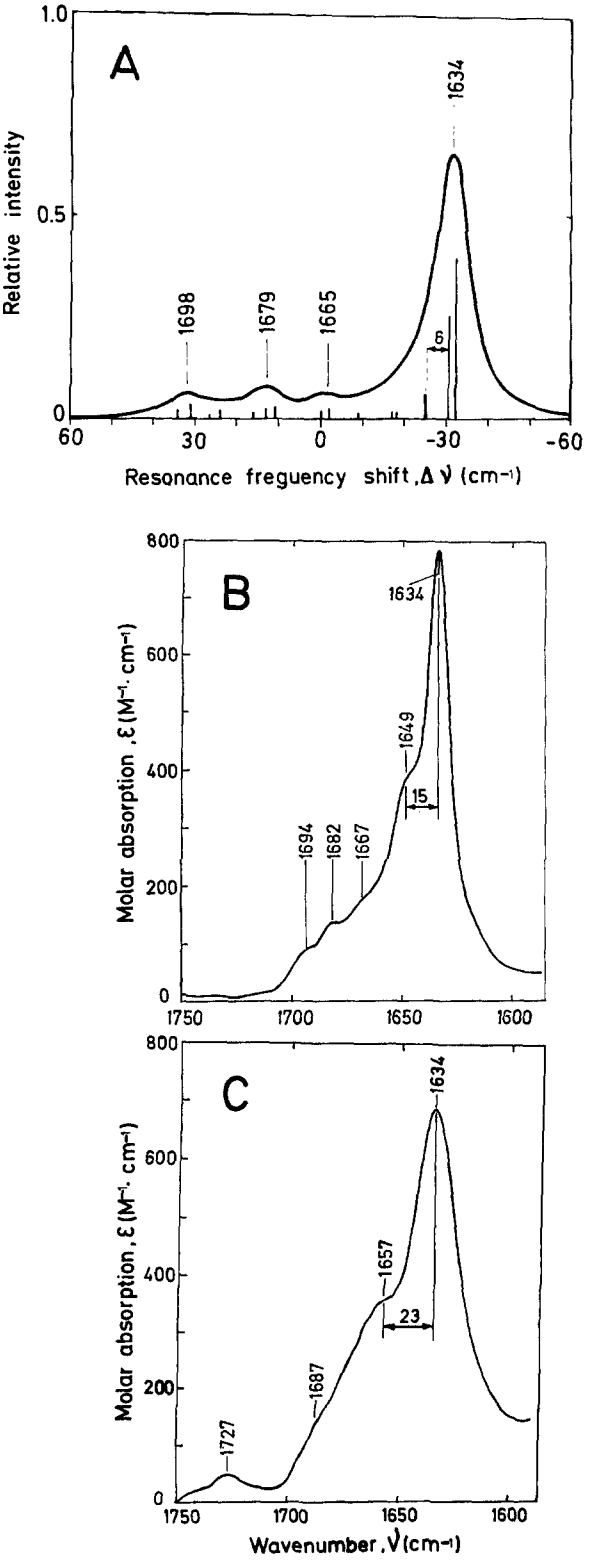

Fig. 12A–C. Computed IR spectra of amide I vibration in ↑↓ππ5.6 helix **A** and observed IR spectra of GA in dioxane solution **B** and in soybean PC liposomes **C**

ππ5.6 helices in solution and in the membrane

FTIR spectra of ππ5.6 helices in solution and in the membrane are similar but not identical (Fig. 12B and C). In the case of unsaturated lipids the shoulder has the

same relative intensity as in the spectrum of solution, but splitting $\Delta\nu$ sh = 23 cm^{-1} is more pronounced than in solution where it is 15 cm^{-1}. Although the reliable calculation is available only for ↑↓ππ helices, the analysis based on the coordinates of the "ideal" ↑↓ππ5.6 helix (obtained by optimization of poly LD alanine) predicts only 6 cm^{-1} splitting in this region (Fig. 12A). These data suggest that distortion of the ππ5.6 helical structure is more pronounced in the membrane than in solution. The same conclusion follows from the increase in the half-width of the amide I band in the membrane spectra in comparison with that in solution ($\Delta\nu_{1/2}$ of the main component are 28 and 13 cm^{-1} respectively). The structural distortions of the helix are probably located in its terminal parts, e.g. due to formation of hypothetical double helix – π4.4 helix hybrid fragments discussed below (Sychev et al. 1980; Heitz et al. 1986).

Putative structures of the GA channel

The experimental data allows us to conclude that in "traditional" bilayer membranes made of monoglycerides, DOPC or egg PC the major contribution to the overall transmembrane GA ion channel current is provided by $\overrightarrow{\pi 6.3}\,\overleftarrow{\pi 6.3}$ helical channels (e.g. Sawyer et al. 1990). Stabilization of this structure by Cs^+ and K^+ cations observed in this work apparently explains the 4–6-fold increase of the channel lifetime with increasing Cs^+ or K^+ ion concentration (Kolb and Bamberg 1977; Ring and Sandblom 1983).

The results obtained here provide more support for the earlier view (Ivanov and Sychev 1983) that the double helix may also form an active transmembrane channel and the ratio of $\overrightarrow{\pi 6.3}\,\overleftarrow{\pi 6.3}$ vs ππ conformers depends on the membrane lipid composition. Theoretical studies of Sung and Jordon (1988) also demonstrated that the double helix can exhibit two binding sites just as well as the head-to-head dimer. Further studies on black lipid membranes are required to provide information on the structure of conducting channels and the influences of alkali metal ions on conformation. It is noteworthy that according to high resolution X-ray data (Langs 1988) the ↑↓ππ5.6 helix has an average inner channel diameter of 4.80 Å, i.e. it is greater than was assumed earlier from molecular models (Veatch et al. 1974) or theoretical analysis (Lotz et al. 1976).

Channels of different structures can be activated and closed by different mechanisms. In the model depicted in Fig. 13, onset and decay of the channel (c–d) are coupled to a massive rearrangement of the hydrogen bonded systems which, at first sight, seems improbable. However, a similar transition ("sliding") between species 1 and 2 in solution (Fig. 14) proceeds with a high rate (Veatch and Blout 1974). One should also take into account that the very slow double helical dimer – monomer equilibrium of GA in non-polar solvents (Veatch and Blout 1974; Sychev et al. 1980) is dramatically facilitated in the presence of phospholipid, as shown by quantitative analysis of HPLC data (Braco et al. 1986).

The GA state shown in Fig. 13d is a hybrid structure, representing partial unwinding of the helix with concomitant formation of terminal non-conducting π4.4 segments. A similar structure was proposed for the gramicidin M^- (enantio 9,11,13,15-Phe-GA) structure in chloroform solution (Heitz et al. 1986).

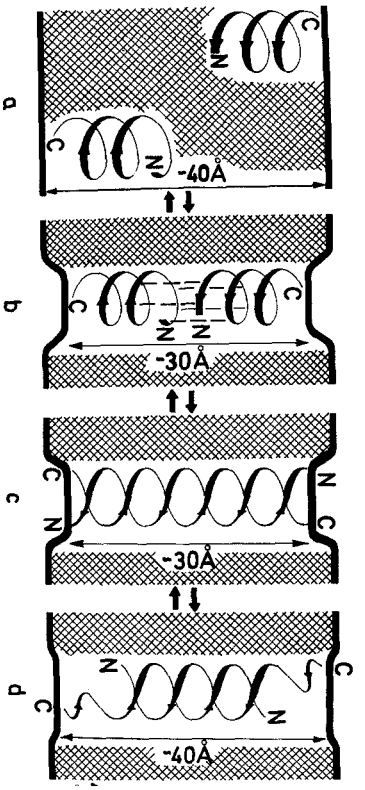

Fig. 13. Switching on and off the single stranded **a, b** and antiparallel double stranded **c, d** helical GA channels

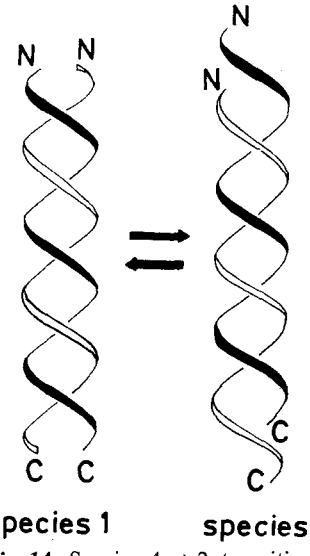

Fig. 14. Species 1 ⇄ 2 transition in left-handed ↑↑ππ5.6 helix. (structures established by Barsukov IL et al. 1987a)

In the present work we found conditions favouring formation of double helices of GA in membranes. At present, we are studying conducting properties of membranes containing such structures.

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