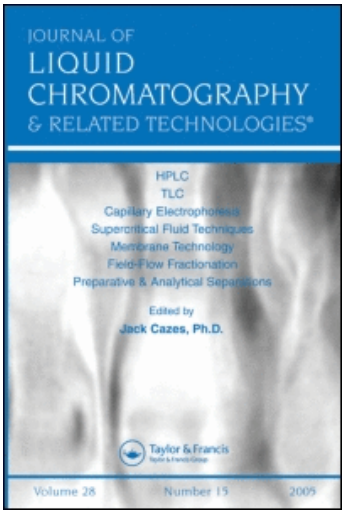

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Membrane Lipid-Polypeptide Molecular Associations in Non Aqueous Solvent. Effect of Phosphatidylcholine Concentration and Temperature and their Influence on the Gramicidin a Dimer-Monomer Conformational Equilibrium

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MEMBRANE LIPID-POLYPEPTIDE MOLECULAR ASSOCIATIONS IN NON AQUEOUS SOLVENT. EFFECT OF PHOSPHATIDYLCHOLINE CONCENTRATION AND TEMPERATURE AND THEIR INFLUENCE ON THE GRAMICIDIN A DIMER-MONOMER CONFORMATIONAL EQUILIBRIUM

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ABSTRACT

The use of an Ultrastyragel 500 Å column for the study of interactions between phosphatidylcholine and gramicidin A in tetrahydrofuran is described. Analysis of vacant peak has allowed to establish the influence that eluent lipid composition, concentration of injected gramicidin and temperature have on the interaction. At 20 °C, for the assayed phospholipid concentration range (0.04 to 0.14 %, w/v), the lipid/polypeptide molar binding ratio, BR, varies from 1.3 to 6.9. An increase in temperature from 20 to 40 °C causes a decrease in BR of about 20 %. On the other hand, the interaction releases some of the water bound to the lipid polar head, suggesting that the binding involves at least this phospholipid moiety. It has been also found that the lipid alters the gramicidin dimer-monomer conformational equilibrium resulting in a higher extent of monomerization, more pronounced as temperature increases. Additional spectrofluorometric measurements are consistent with the experimental evidence deduced from HPLC analysis.

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INTRODUCTION

Gramicidin A (GA) is a linear hydrophobic antibiotic polypeptide HCO-LVal-Gly-LAla-DLeu-LAla-DVal-LVal-DVal-LTrp-DLeu-LTrp-DLeu-LTrp-DLeu-LTrp-$NHCH_2CH_2OH$, which forms ion channels in biological and artificial membranes (1). Structural studies of GA incorporated into liposomes have confirmed its dimeric nature (2-4) although the specific conformation of GA in the conducting transmembrane channel (head to head dimers, parallel and antiparallel double helices, β-sheet conformation...) is at present a current matter of controversy (5-8). It is clear that the peptide to lipid ratio seems to be an important parameter influencing the conformational state of GA in these systems (5,8,9).

On the other hand, detailed comparison of the experimental and computerized ir spectra and CD measurements in nonpolar solvents has shown that the dimeric gramicidin species are predominantly antiparallel double helices (6,10-12) in equilibrium with smaller amounts of head to head associated π_{LD} helices (12). It is also known that in certain conditions, the same GA conformation seems to predominate in a nonpolar solvent (such as dioxane or chloroform) as in dipalmitoylphosphatidylcholine liposomes at 20 °C and low concentrations of the antibiotic (5,8).

Model HPLC experiments with GA in tetrahydrofuran (THF)-phosphatidylcholine (PC) mixtures have pointed towards a strong interaction between GA and the lipid (13,14), that has been quantified in terms of binding ratio parameter (14). We describe in this paper our results concerning the application of Ultrastyragel 500 Å for the analysis of PC-GA interaction in THF. The effect of lipid eluent composition, concentration of injected polypeptide and temperature have been investigated. It will be shown that lecithin and GA interact in such a manner that the environment of the tryptophanyl (trp) indole ring becomes more polar with a simultaneous release of some of the solvation water originally constrained around the lipid polar head. At the same time, the presence of phospholipid

and an increase in temperature seem to alter the GA conformational equilibrium towards monomeric forms in a similar way to that observed in more polar solvents (10).

MATERIALS

Reagents

GA was supplied by Koch Light Lab. and was used in all the experiments without further purification. Egg yolk PC was purified by preparative column chromatography according to Singleton et al. (15), the collected fractions were analyzed by TLC and those containing only PC were pooled together, filtered through a 0.45 μm Micro Filtration Systems filter and concentrated under vacuum in a rotary evaporator. The PC was then dissolved in absolute ethanol and concentrated in order to remove water. This step was repeated twice more and additional lyophilization step was omitted so that lecithin retained a minimum amount of water allowing its total solubilization in THF. PC was kept under nitrogen at -20 °C until use.

All the solvents used in PC purification (chloroform, methanol, absolute ethanol) were analytical reagent grade. THF was a Merck spectroscopic reagent and was degassed under vacuum prior to use.

Liquid Chromatography Instrumentation

A Waters Associates Liquid Chromatographic System equipped with a model M-45 solvent delivery unit, a model U6K universal injector and an Ultrastyragel 500 Å column was used. Samples were always simultaneously monitored with a model R401 differential refractometer, from Waters Assoc. and a Varian Varichrom variable wavelength UV-VIS detector, set at 290 nm. The chromatograms were recorded using a Yokogawa Electric Works dual channel recorder. In all the experiments, temperature control was performed by immersing the column in a Heto Ultrathermostat water bath.

Spectrofluorometry

Fluorescence experiments were performed in a Perkin Elmer MFP-44B spectrofluorometer with automatic corrector of excitation or emission spectra. Excitation was at 297 nm and emission spectra were recorded up to 400 nm, excitation and emission slits being 4 and 8 nm respectively. Temperature was controlled by thermostatizing the cell at 20 °C with a Lauda circulating water bath.

METHODS

Equilibrating Conditions

The column was isocratically equilibrated with binary PC solutions, each one prepared by dissolving in THF a weighed amount of PC, filtering the solution through a 0.45 μm MFS regenerated cellulose filter and then completing the volume to 1 L. The concentrations of the equilibrating PC solutions used ranged from 0.02 to 0.14 % (w/v). All the experiments were carried out at a constant flow rate of 1.0 mL/min (≃ 500 psi). Column temperatures ranging from 20 to 50 °C were used. The column was allowed to equilibrate for at least 30 min at each assayed temperature before injecting any GA sample, and no baseline drift was observed.

As we have previously reported (14), PC interacts very strongly with the polystyrene-divinylbenzene reticular matrix of the gel. On the other hand, we have observed that in order to achieve a good separation between PC and GA the support must be essentially devoid of lipid, that is, the column must be regenerated after each equilibration experiment. Since we have also checked that heating improves removal of PC from the gel, a systematic clean up procedure was adopted between two assayed eluent compositions consisting of eluting the column with THF about 40 °C for 3 or 4 days, what allowed to wash off the PC and to prevent any interference in resolution due to former equilibration experiments.

Sample Injection

GA samples were prepared immediately before injection always at room temperature, irrespective of column temperature, by dissolving the polypeptide in the binary equilibrating solution. The injected volume was in all cases 50 μL and the GA concentration ranged from 0.2 to 10.0 mg/mL.

For each eluent composition and for each assayed temperature, several volumes of THF were injected corresponding to known defects in absolute amount of PC, $\Delta m°$, in mg. A calibration plot of $\Delta m°$ vs A° was so obtained, A° being the area of defect peak appearing in the chromatogram. Subsequent injection and elution through the column of the GA sample caused a PC defect vacant peak of area A. The Δm value corresponding to the area A was deduced from the calibration plot as previously described (13,14).

Fluorescence Experiments

The intrinsic fluorescence emission spectra were obtained at 20 °C by adding several aliquots, up to 50 μL, of a 300 mg/mL phospholipid in THF solution, directly into a cuvette containing 3 mL of GA solution. Different GA concentrations were assayed.

RESULTS AND DISCUSSION

In the present work the interaction of PC with GA in THF has been studied in binary eluents of compositions 0.02, 0.04, 0.06, 0.08, 0.12 and 0.14 % (w/v). Figure 1 shows as an example the chromatograms obtained for an injected GA sample of 50 μL at a concentration of 2.0 mg/mL, for a 0.08 % (w/v) PC in THF solution as eluent at 20, 30, 40 and 50 °C. The chromatograms observed at 20 °C are similar to those previously described (14). The peak eluting at 6.5 mL corresponds to GA solvated by lipid (peak D) and the one eluting at 8.0 mL to the defect vacant peak (peak V). In all cases, a minor peak appearing at 6.1 mL is observed (peak M).

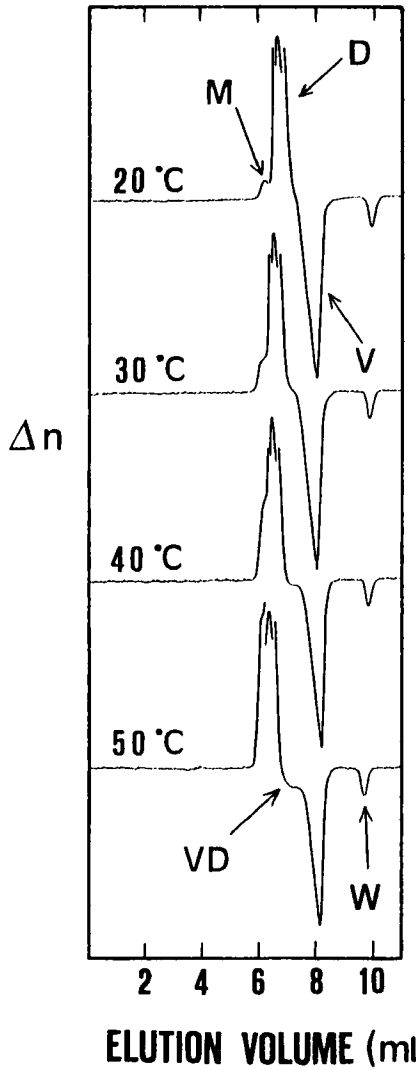

FIGURE 1. Elution profiles of GA for a 0.08 % (w/v) PC in THF solution as eluent at 20, 30, 40 and 50 °C. GA concentration was 2.0 mg/mL. The samples were monitored by refractive index detector. The injection volume was 50 µL in all cases. W, corresponds to the peak of water excess.

An increase in temperature gives rise to the following effects: A) an increase in the elution volume of peak V, also evident in calibration chromatograms, due to a lower adsorption of the lipid on the gel, and a decrease in peak D elution volume, originated by a mechanical effect of the support (the same behavior is observed for GA in pure THF as eluent), which causes a better resolution of both peaks. It is known that several variables can be involved in the effect that an increase in column temperature has on retention volume. In our case, the most significant ones could be an expansion in volume of the gel, gel-solute and gel-solvent interactions and hydrodynamic volume of solute (16). B) an increase in the area of peak M, accompanied by a larger overlapping with peak D. C) the appearance of a slight distortion or depression in the vacant peak (depression VD) which becomes quantitatively more important as temperature rises. The increase in the depression VD area seems to be related to the increase in peak M area.

As mentioned above, the presence of lipid in the eluent results in the appearance of a minor peak as it can be deduced from RI, and more selectively UV responses. However, this peak is absent when GA is injected at the same concentration in pure THF as eluent. In figure 2 the chromatograms obtained at 20 °C for eluent lipid compositions 0.04, 0.06, 0.08 and 0.12 % (w/v) and for GA without lipid are compared. The contribution of these minor peaks accounts for only about 1-2 % of total GA for the most diluted eluents and up to 8-10 % for the most concentrated ones.

In a similar manner as shown in figure 1, it has been verified, for all the eluents assayed, that for a given injected GA concentration an increase in temperature causes an increase in peak M to peak D ratio.

On the other hand, the variation of vacant peak area as a function of injected GA concentration has been studied for each eluent at 20 °C. The absolute amount of PC defect, in mg, which represents the lipid interacting with GA, has been calculated from

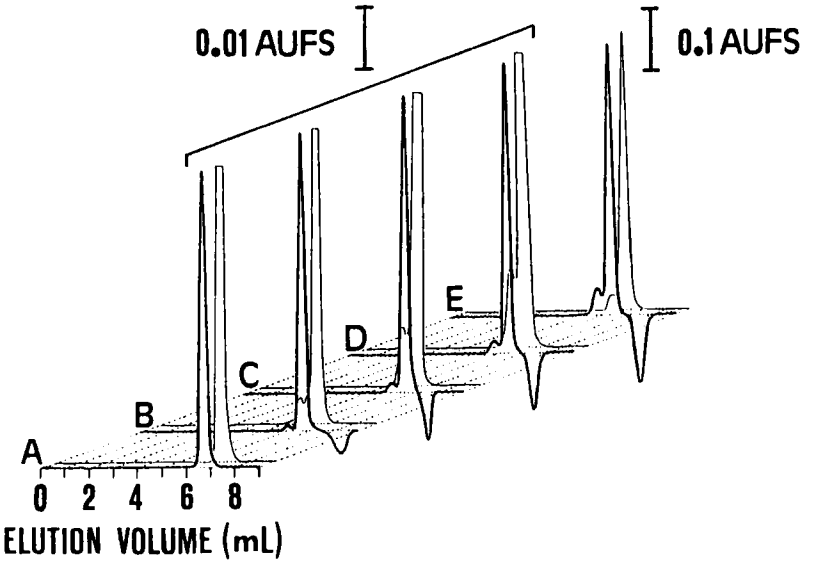

FIGURE 2. Normalized chromatograms obtained for GA in eluents of different PC compositions monitored simultaneously with a differential refractometer (——) and UV detector at 290 nm (——). Eluent lipid concentrations were, in % (w/v): (A) 0, THF as eluent; (B) 0.04; (C) 0.06; (D) 0.08 and (E) 0.12.

vacant peak areas as described in Methods. The results obtained for a 0.08 % (w/v) PC in THF solution as eluent are shown in figure 3A. A linear relationship is observed only at low GA concentrations, up to 2.0 mg/mL, whereas at higher GA concentrations an upwards deviation occurs indicating more probably a lipid-polypeptide binding equilibrium rather than a simple preferential solvation phenomenon.

Lyophilized, highly dehydrated PC is a very hygroscopic product not directly soluble in pure THF. A small amount of water must be added to this organic solvent in order to solubilize the phospholipid. This fact indicates that PC molecules retain some hydration water presumably bound to their polar heads, when they

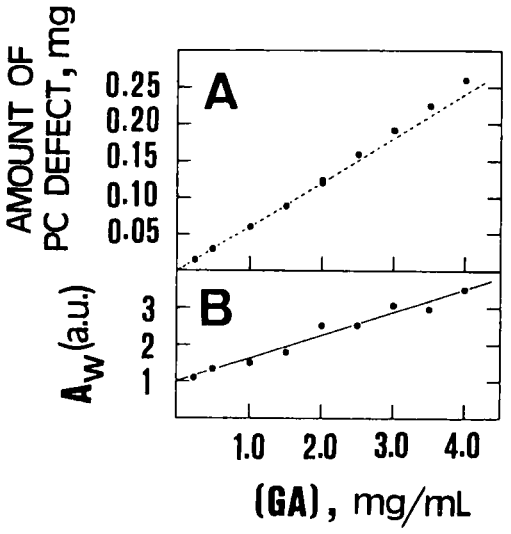

FIGURE 3. Dependence of (A) the absolute amount of PC defect in mg and (B) the area of water excess peak, A_w, in arbitrary units, vs injected GA concentration for a 0.08 % (w/v) PC in THF solution as eluent.

are solubilized in THF. This requirement, however, instead of being an additional problem complicating the system, has proved to be of interest for elucidation of some aspects of the interaction. In fact, a peak of water excess is observed in chromatograms corresponding to this eluent whose area increases proportionally with increasing injected GA concentration (figure 3B). This suggests that GA-PC interaction releases water originally bound to the lipid polar head. This observation could be related to recent membrane fusion studies in artificial vesicles which suggest that the key event in the actual fusion reaction is a localized dehydration at the site of membrane contact (17). On the other hand, Yoshida et al. (18) have pointed out that inhalation anesthetics cause a release of bound water when acting on water-in-oil emulsions and postulated that dehydration of the interface would interfere with the transport of current carrying hydrated

TABLE 1

BR Values Calculated from Vacant Peak Areas for Different Eluent PC Concentrations at Several Temperatures.

Eluent PC concentration % (w/v)	Temp. (°C)	GA (mg/mL)	Absolute amount of PC defect, mg	PC defect, mg / inj. GA, mg	BR
0.02	20		no vacant peak *		
0.04	20	1.0	0.02_8	0.55	
		2.0	0.04_4	0.44	1.3 ± 0.2
		3.0	0.08_9	0.59	
0.06	20	1.0	0.03_7	0.73	
		1.5	0.05_7	0.76	1.9 ± 0.1
		2.0	0.08_2	0.82	
		3.0	0.12_6	0.84	
	25	1.0	0.03_2	0.64	
		1.5	0.04_7	0.63	1.6 ± 0.1
		2.0	0.07_0	0.70	
	30	1.0	0.02_9	0.58	
		1.5	0.05_1	0.68	1.6 ± 0.1
		2.0	0.06_8	0.68	
	35	1.0	0.03_2	0.63	
		1.5	0.04_7	0.62	1.46 ± 0.07
		2.0	0.05_6	0.56	
	40	1.0	0.02_7	0.54	
		1.5	0.04_5	0.60	1.4 ± 0.1
		2.0	0.06_3	0.63	
0.08	20	0.5	0.02_9	1.16	
		1.0	0.06_0	1.20	
		1.5	0.08_8	1.17	2.86 ± 0.07
		2.0	0.12_1	1.21	
		2.0	0.12_1	1.21	
	30	2.0	0.10_8	1.08	
		2.0	0.11_3	1.13	2.66 ± 0.05
		2.0	0.11_2	1.12	
	40	2.0	0.10_5	1.05	
		2.0	0.09_8	0.98	2.4 ± 0.1
		2.0	0.10_0	1.00	
	50	2.0	0.09_1	0.91	
		2.0	0.08_9	0.89	2.2 ± 0.1
		2.0	0.09_7	0.97	

TABLE 1 (cont.)

0.12	20	1.0	0.10_0	2.00	5.0 ± 0.2
		2.0	0.21_0	2.10	
		2.5	0.25_3	2.02	
		3.0	0.33_1	2.20	
	40	1.0	0.09_9	2.00	4.6 ± 0.1
		2.0	0.19_1	1.91	
		3.0	0.28_7	1.92	
		4.0	0.37_8	1.89	
0.14	20	0.3	0.04_7	3.16	6.9 ± 0.5
		0.5	0.07_0	2.80	
		0.5	0.07_2	2.88	
		1.0	0.14_0	2.80	
		1.5	0.20_6	2.75	
	40	0.2	0.02_8	2.82	5.3 ± 0.9
		0.3	0.04_0	2.70	
		0.6_5	0.07_0	2.15	
		1.0	0.09_7	1.94	
		1.2_5	0.13_5	2.16	
		1.5	0.15_9	2.12	

* At this eluent PC composition, the support is probably not equilibrated, what causes some instability of the chromatogram baseline and no measurable vacant peak area.

ions through membranes and may constitute a molecular mechanism of anesthesia.

Table 1 summarizes the results at different temperatures for GA samples injected at several concentrations for eluent compositions ranging from 0.02 to 0.14 % (w/v). From these data the molar binding ratio parameters, BR, have been calculated for each eluent as previously described (14). The BR parameter increases with increasing eluent PC concentration up to a value of 6.9 moles PC/mole GA for a 0.14 % (w/v) eluent composition at 20 °C.

The variation of this parameter at 20 and 40 °C as a function of eluent PC concentration is shown in figure 4. The figure includes the experimental BR value obtained at 20 °C for a 0.10 % (w/v) eluent concentration (14), which is in good agreement

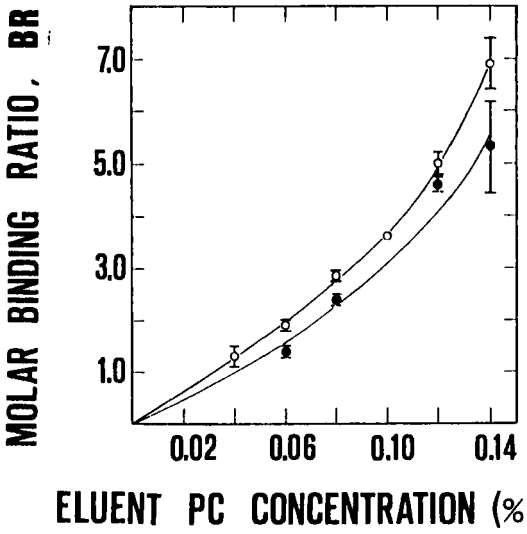

FIGURE 4. Molar binding ratio parameter, BR, vs eluent PC composition expressed in % (w/v), at 20 °C (○) and 40 °C (●). Each point is the mean value ± its average deviation.

with the present data. Experiments with PC eluent concentrations higher than 0.14 % (w/v) have been performed but resolution between peak D and peak V is poor and quantitation is not possible.

If it is assumed that the system presents cooperativity, the experimental values can be fitted to the first section of a sigmoidal curve and by means of Hill graphical plots an estimation of the number of binding sites, n, and an overall association constant, K_a, can be obtained. Approximate values of 20-30 for n and about 10^4-10^5 M^{-1} for K_a have been determined.

The variation of vacant peak areas has been studied in a similar way for each eluent as a function of temperature for several given GA concentrations. The results obtained for eluents of compositions 0.06 and 0.08 % (w/v) are shown in figure 5. The normalized absolute PC defects per milligram of GA slightly decrease with increasing temperature; this indicates a lower extent of inter-

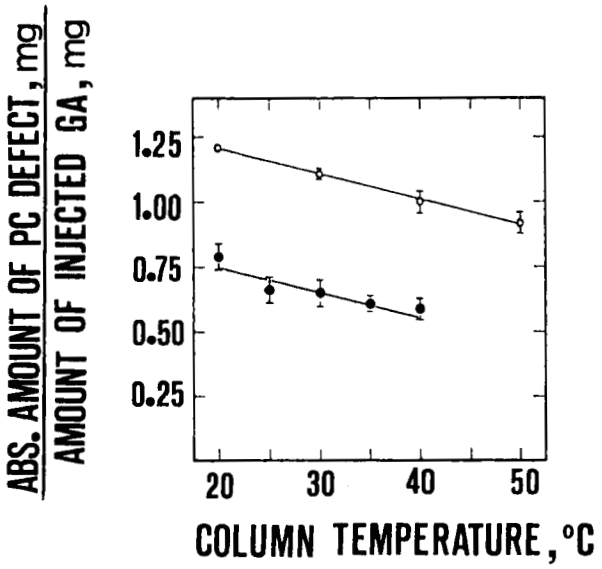

FIGURE 5. Effect of temperature on the normalized absolute amount of PC for eluents of different PC composition. (●) 0.06 and (○) 0.08 % (w/v) PC in THF solution as eluent. Each point is the mean value ± its average deviation.

action at higher temperatures. Brophy et al. (19) have shown in artificial vesicles a relatively small temperature dependence of the myelin proteolipid protein-phospholipid interactions over the range 30-40 °C, suggesting a relatively high degree of motional restriction for the protein-interacting lipids. In our case, the fact of existing a more significant temperature dependence, though also small, may be due to a higher degree of motional freedom of bound PC in the organic solvent. As it was mentioned above, an increase in temperature causes a depression of vacant peak (fig. 1) and, on the other hand, PC-GA interaction releases solvation water (fig. 3B). Figure 6 evidences these changes by comparison of chromatograms obtained at 40 °C for an eluent composition of 0.12 % (w/v) as a function of injected GA concentration. The vacant peak depression VD also increases with increasing GA concentration. In addition,

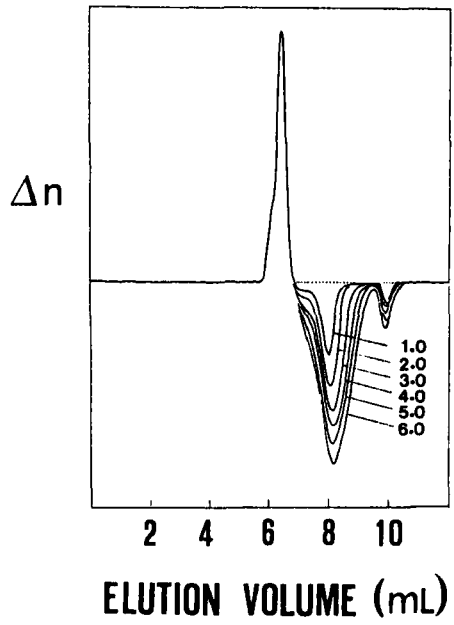

FIGURE 6. Chromatograms showing vacant peak, vacant peak depression and peak of water excess obtained at 40 °C by injection of 50 μL aliquots of GA samples at different concentrations. Eluent: a 0.12 % (w/v) PC in THF solution. The numbers in the figure correspond to injected GA concentration expressed in mg/mL. In order not to overcrowd the figure only the peak of solvated GA corresponding to 1.0 mg/mL is included.

the comparison of chromatograms corresponding to the same GA concentration and the same temperature has allowed to establish that the area of depression VD increases with eluent lipid concentration (see figs. 1 and 6).

It is known that GA exists in nonpolar solvents such as dioxane or ethylacetate as a family of interconverting dimers organized as intertwined double helices aligned predominantly in an antiparallel form and stabilized by hydrogen bonds (5,10-12). The transition of GA dimer to monomer is a very slow process in these solvents, the monomeric species being a π_{LD} helix (12).

Commercial gramicidin used is a crystalline powder of dimeric structure. Since all injections were performed immediately after dissolving the polypeptide in the equilibrating solution, GA probably adopts in pure THF a dimeric structure similar to that described for nonpolar solvents (peak D). The presence of phospholipid in the eluent alters the GA conformational equilibrium so that an increasing dissociation of the original dimer to a monomeric form (peak M in chromatogram) occurs as the lipid eluent composition is raised. Since the lipid is an amphiphilic molecule, it is not surprising that GA conformational equilibrium be altered similarly as in more polar solvents (10,11).

We have verified (data not shown) that the lipid does induce dimer to monomer transition by injecting mixed solutions of GA and PC in THF at different PC concentrations into an Ultrastyragel 1000 Å column equilibrated with pure THF, and that the displacement towards monomeric forms is higher as PC/GA ratio increases. This fact is in agreement with the behavior of peak M as a function of lipid eluent composition (fig. 2). However, peak M appears in the chromatograms at an elution volume lower than the corresponding to its molecular weight, that is, elution volumes for dimer and monomer seem to be inverted in relation to their molecular weight and to their order of elution on Ultrastyragel 1000 Å. Some possible non-exclusive explanations can be given:

a) a higher lipid solvation for two monomeric species than for a dimeric one as evidenced by the appearance of vacant peak depressions VD due to monomerization along the column. A differential solvation by PC is to be expected based on the different conformations and presumably different accessibility of both species (12), that would give rise to a higher hydrodynamic volume for the lipid-monomer complex. In this connection, it has been extensively described that in solvent/solvent/polymer systems a decrease in polymer molecular weight results in an increase of preferential solvation (20,21). As to the depression VD is concerned, Berek et al. (22) have described that resolvation phenomena along a chromatographic column cause distortions of vacant peak.

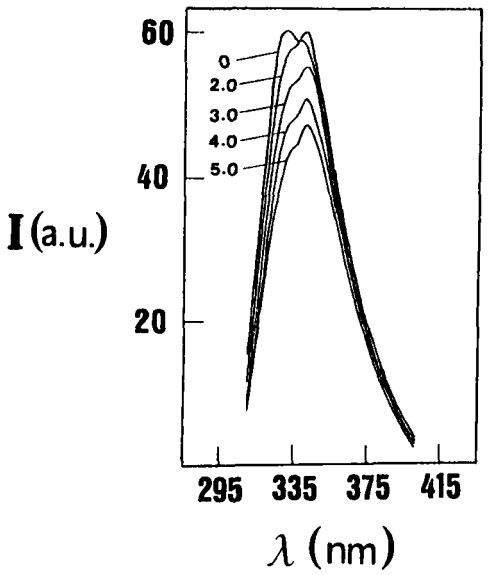

FIGURE 7. Effect of addition of PC on the emission spectrum of GA at a concentration of 0.05 mg/mL. The numbers in the figure indicate the PC concentrations in the cuvette expressed in mg/mL. Each emission spectrum was recorded immediately after adding the corresponding lipid solution aliquot and stirring for about 10 seconds for complete mixing.

b) since the phospholipid strongly interacts with gel matrix, the lipid solvated GA dimeric form could be more retained than the corresponding solvated GA monomer.

On the other hand, it has been observed by injecting GA samples at different concentrations in pure THF as eluent that an increase in temperature from 20 to 40 °C does not originate any minor peak at the elution volume of GA monomer. However, for any given eluent PC concentration an increase in temperature gives rise to an appreciable increase in peak M (see fig. 1). This can be attributed to the fact that increasing the temperature accelerates the monomerization process in the presence of lipid.

As a conclusion, the two factors mentioned above, that is, lipid eluent concentration and temperature, alter the dimer-monomer conformational equilibrium of GA producing a stimulating effect in the dissociation rate.

In addition to HPLC-SEC chromatographic analysis, we have also used the fluorescence characteristics of the tryptophanyl residues of GA as a probe of binding phenomena. In THF, GA fluorophores show an emission spectrum with two maxima at 330 and 338 nm (figure 7), due to vibrational structure displayed by the indole ring when dissolved in a nonpolar and aprotic solvent (23). Upon addition of lecithin both a red shift and a decrease in fluorescence intensity (quenching due to complex formation) occur (fig. 7). The shift towards higher wavelength indicates a more polar environment, that confirms the involvement of the lipid polar head, at least, in the interaction with the polypeptide. This assumption is supported by the chromatographic observation that water bound to the phospholipid is released in this interaction. In the light of these results, it may be worth studying the effect of PC on the kinetics of the conformational equilibrium of gramicidin by using other SEC columns with improved resolution for GA dimeric and monomeric species and by applying alternative methodologies.

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