Journal of Chromatography, 353 (1986) 181–192
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMSYMP. 776

TIME-DEPENDENT MONOMERIZATION OF GRAMICIDIN A, ENHANCED BY PHOSPHATIDYLCHOLINE IN NON-POLAR SOLVENTS

A HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC AND SPECTROFLUOROMETRIC STUDY

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SUMMARY

The usefulness of size-exclusion high-performance liquid chromatography for the study of gramicidin A dimer–monomer conformational equilibrium in non-polar solvents is demonstrated for the first time. The separation of dimeric and monomeric species on an Ultrastyragel 1000 Å column was optimal, and the monomerization process was analysed in the presence and absence of phosphatidylcholine as a function of time. The phospholipid alters the polypeptide conformational equilibrium. Higher lipid concentrations result in a more complete monomerization. Simultaneous high-performance liquid chromatographic and spectrofluorometric kinetic studies have revealed that the monomerization process is accompanied by enhancement of the fluorescence emission intensity, which could be attributed, at least in part, to different fluorescent features of the monomer relative to the dimer. The molar ratio of the lipid to the polypeptide is also an important parameter influencing the state of the system. A model for interactions between gramicidin A and phosphatidylcholine in tetrahydrofuran is proposed, in which the lipid enhances the dissociation of the dimer and stabilizes the monomeric forms.

INTRODUCTION

Since the proposal of gramicidin A (GA) dimeric channel independently by Ramachandran and Chandrasekaran[1] and by Urry *et al.*[2,3], efforts have been made to determine its specific conformation. GA is a linear polypeptide antibiotic, consisting of fifteen alternating L- and D-hydrophobic amino acid residues[4], which facilitates the passive diffusion of monovalent cations through natural and artificial lipid bilayer membranes. A number of different dimeric helices are possible, owing to the alternating sequence of L- and D-residues[5,6].

Based on previous studies on model-building and spectroscopic measurements

0021-9673/86/$03.50

in organic solvents, the existence of an N-terminal to N-terminal π_{LD} helix has been proposed[2,3]; this is supported by conductance studies of lipid membrane bilayers[7]. On the other hand, the conformation of GA in organic solvents deduced from IR[8], NMR[9] and CD[8,10,11] studies established that the polypeptide exists as a family of interconverting dimers, organized as intertwined double helices aligned predominantly in an antiparallel form. The dimerization constant in relatively non-polar solvents, such as dioxane and ethyl acetate, is much higher than in more polar ones, such as methanol and dimethyl sulphoxide; this suggests that the dimers are stabilized by hydrogen bonds. In fact, decreasing solvent polarity strongly favours the aggregated species having a large enough stability to allow their isolation by physical techniques[12].

The conversion of the GA dimer into monomer is a very slow process in non-polar solvents: it takes over twenty days for the conformational equilibrium in dioxane to become established[8,10]. The monomeric species has been identified as a single π_{LD} helix[8]. On the contrary, a monomeric form with little or no secondary structure is favoured in more polar solvents[10,12], where the conformational equilibrium is attained in a much shorter time (*e.g.*, 2–4 h in methanol).

Although structural studies of GA, incorporated into liposomes, have also confirmed the dimeric nature of the GA channel, the specific conformation of GA is still a matter of controversy[11,13,14].

High-performance liquid chromatography (HPLC) has recently been used in our laboratory to characterize lipid–lipid and lipid–polypeptide interactions in non-aqueous solvents[15,16]. Analysis of vacant peaks with an Ultrastyragel 500 Å column revealed that phosphatidylcholine (PC) strongly binds to GA and also alters the polypeptide dimer–monomer conformational equilibrium[17]. In the past few years size-exclusion HPLC techniques have been applied to the study of dissociation and recombination of large proteins, *e.g.*, the time-dependent monomerization of bacteriorhodopsin induced by Triton X-100[18], the extent of dissociation of human chorionic gonadotropin subunits as a function of time and temperature[19], etc. The kinetics of proteolysis of medium-molecular-weight polypeptides, such as insulin, has been also reported[20].

In the present study, we have examined a new steric exclusion column, Ultrastyragel 1000 Å, for the analysis of the dissociation of GA dimers in organic solvents in the presence and absence of a phospholipid, PC, at different molar ratios of lipid to polypeptide, as a function of time. Total resolution of both GA dimeric (MW *ca.* 3700) and monomeric (MW *ca.* 1850) species has been achieved for the first time, so that it is now possible to visualize directly the time-course of the GA conformational equilibrium, which has been previously studied in liposomes and in organic solvents basically by spectroscopic techniques.

EXPERIMENTAL

Apparatus

The liquid chromatograph consisted of an M-45 solvent-delivery system and an U6K universal injector from Waters Assoc. (Milford, MA, U.S.A.). Detectors used were a Varian Varichrom variable-wavelength UV–VIS detector (Varian Aerograph, Walnut Creek, CA, U.S.A.) and a Waters Assoc. R-401 refractive index de-

tector. The system was equipped with a 100-nm pore-size Ultrastyragel column (30 × 0.78 cm I.D.) from Waters Assoc. The chromatograms were recorded by using a Yokogawa Electric Works dual-channel recorder (Tokyo, Japan).

Fluorescence experiments were performed in a Perkin-Elmer MPF-44B spectrofluorometer (Überlingen, F.R.G.) with automatic correction of excitation or emission spectra. Excitation was at 297 nm, and emission spectra were recorded up to 400 nm; the excitation and emission slits were 4 and 8 nm, respectively. The temperature was controlled within ± 0.1°C by using a Lauda Compact Thermostat MT-20 (Lauda, Koningshofen, F.R.G.).

Chemicals and reagents

Gramicidin A was supplied by Koch Light (Buckinghamshire, U.K.) and was used without further purification. Egg yolk phosphatidylcholine was purchased from Merck (Darmstadt, F.R.G.) and purified according to the column chromatographic method of Singleton *et al.*[21]. The phospholipid gave a single spot when analysed by thin-layer chromatography. Tetrahydrofuran was spectroscopic grade (Merck). It was passed through a 0.45-μm Micro Filtration Systems regenerated cellulose filter (Dublin, CA, U.S.A.) before use.

Procedures

All GA samples were prepared in tetrahydrofuran and stirred for 1 min immediately after preparation for complete mixing. Tightly stoppered 10-ml glass tubes were completely filled with the corresponding solutions to minimize hydration, and stored in a dark room at 25°C until injection.

All chromatographic experiments were conducted at room temperature. The column was eluted isocratically with tetrahydrofuran at a flow-rate of 1.0 ml/min (*ca.* 20.7 bar). The injection volume was variable so that the absolute amount of injected GA for all the assayed concentrations was similar: 5, 10, 25, 20, and 100 μl were injected for GA concentrations of 1.0, 0.5, 0.1, 0.05, and 0.01 mg/ml, respectively. It was previously verified that changing the injection volume within this range did not appreciably alter the peak ratio in the chromatograms.

The intrinsic fluorescence emission spectra were obtained at 20°C by adding several aliquots, up to 50 μl, of a solution of phospholipid in tetrahydrofuran (300 mg/ml) directly to a cuvette containing 3 ml of GA solution. GA concentrations ranging from 0.005 to 1.0 mg/ml were assayed. Kinetic experiments by spectrofluorometry were performed at 25°C by measuring the fluorescence intensity at 330 and 338 nm. Other details of the experimental conditions used can be found in the corresponding legends to figures.

RESULTS

Fig. 1 depicts the elution profiles of several samples of GA in tetrahydrofuran in the absence (A) and presence (B) of lipid, injected *ca.* 2 h after solution and after 21 days. Two peaks, completely resolved, are eluted at 7.9 ml (I) and 8.4 ml (II). Peak I decreases with time, whereas peak II increases. This effect is markedly enhanced in the presence of phospholipid. When these peaks are collected and reinjected separately after 24 h, peaks I and II again appear in the chromatograms, indicating

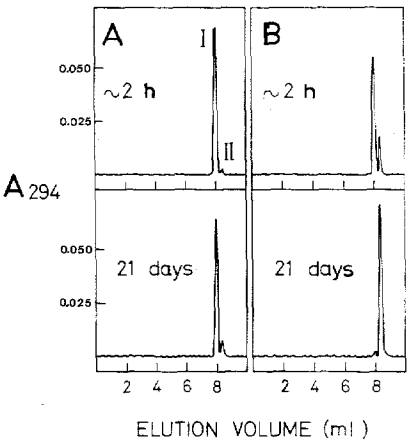

Fig. 1. Elution profiles of GA in the absence (A) and presence (B) of PC, recorded *ca.* 2 h after solution and after 21 days of incubation. The GA concentration was 1.0 mg/ml and the PC concentration was 2.1 mg/ml. Chromatography was carried out at a flow-rate of 1.0 ml/min with tetrahydrofuran as eluent on an Ultrastyragel 1000 Å column. The void volume of the column was 5.0 ml. GA samples were monitored by absorbance at 294 nm and recorded at a chart speed of 1.0 cm/min. The injection volume was 5 μl in all cases.

that two different interconvertible states of the polypeptide exist. We have verified that, in the range of concentrations used, this lipid does not interfere with the resolution of both GA species, because PC strongly interacts with the stationary phase and is eluted later.

It is known that GA is present in different dimeric and monomeric forms in organic solvents, and since the dimer:monomer ratio basically depends on solvent polarity[8,10], a comparative study in several solvents has been carried out in order to

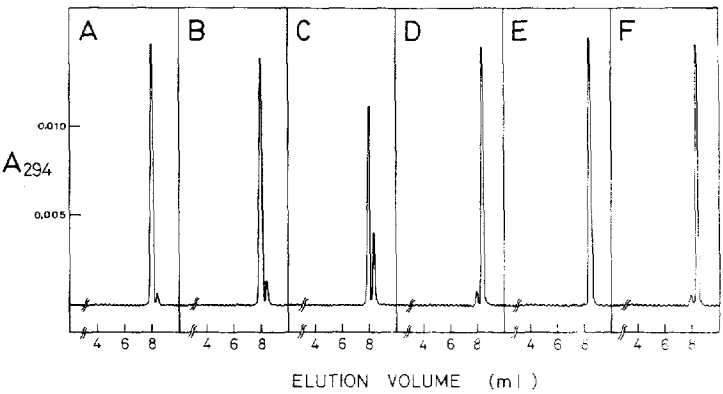

Fig. 2. Elution profiles of GA in (A) dioxane, (B) ethyl acetate, (C) ethanol, (D) methanol, and (E) dimethyl sulphoxide. (A–E) GA samples (0.5 mg/ml) were prepared by dissolving the polypeptide in each solvent, and aliquots of 2 μl were injected 2–3 h after solution into a column equilibrated with tetrahydrofuran. (F) GA lyophilized from acetic acid was dissolved in tetrahydrofuran at a concentration of 0.14 mg/ml, and an aliquot of 25 μl was injected, 20 min after solution. In all chromatograms, the peak eluted at 7.9 ml corresponds to the GA dimer and the peak at 8.4 ml to the monomer.

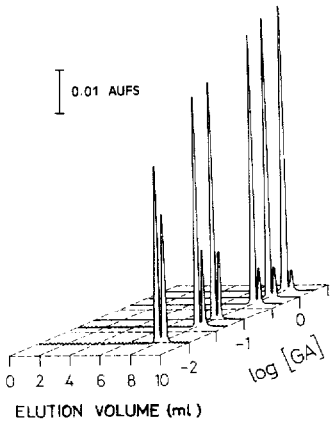

Fig. 3. Elution profiles of different GA concentrations at 21 days. GA concentrations were 0.01, 0.05, 0.1, 0.5, 1.0, and 2.0 mg/ml, and the injection volumes were 100, 20, 25, 5, 5, and 2.5 μl, respectively.

ascertain the identity of GA peaks I and II. The comparison of the chromatographic results (Fig. 2) with data reported by Veatch *et al.*[10] has allowed us to establish that the peak eluted at 7.9 ml corresponds to a GA dimeric form and the peak at 8.4 ml to a monomeric form. Therefore, the HPLC elution peaks in Fig. 1 correspond to a dimer (I) and monomer (II), respectively. It can be assumed that equilibrium is practically achieved within 21 days (Fig. 1A) on the basis of the data from the literature quoted above[8,10]. It is not likely that the equilibrium is altered through the column, because the rate of dimer dissociation at room temperature under the conditions of elution is too slow to cause a significant change in the state of association during the time required for the analysis.

The presence of phospholipid in the solution induces GA monomerization (Fig. 1B), and it will be shown further that this phenomenon is a function of time, lipid concentration and the molar ratio of PC to GA.

Fig. 3 shows the chromatograms of GA samples obtained at different concentrations in tetrahydrofuran after equilibrium was reached. The figure reveals the dependence of the conformational equilibrium on GA concentration, so that the lower the polypeptide concentration, the higher the mass fraction of monomer. From the heights of the dimer and monomer peaks, an association constant for GA in tetrahydrofuran has been estimated, $K_a = 1.1 \cdot 10^5\ M^{-1}$, assuming that the molar extinction coefficient of tryptophanyl (Trp) residues of both species is the same. This value is of the same order of magnitude than those reported for non-polar solvents[8,10]. The conformational equilibrium of GA is a function of time (Fig. 1), and of PC and GA concentrations (Figs. 1B and 3). Therefore, a detailed kinetic study has been carried out at several concentrations of GA, at different PC concentrations.

For example, Fig. 4 illustrates some of the results obtained. Both elapsed time (Fig. 4A) and an increase in the PC concentration (Fig. 4B) result in a displacement of equilibrium towards the monomeric forms, whereas at a fixed PC concentration an increase in the GA concentration reduces the extent of monomerization (Fig. 4C).

Fig. 5 shows as an example the mass fraction of monomer as a function of time for a GA concentration of 1.0 mg/ml at different PC concentrations. An increase

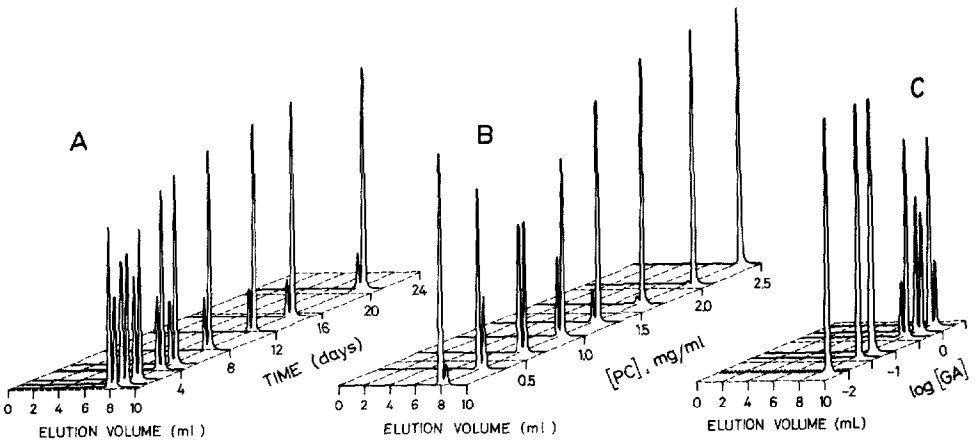

Fig. 4. (A) Time-dependent monomerization of GA at a fixed PC concentration of 1.3 mg/ml; the GA concentration was 1.0 mg/ml, and the injection volume 5 μl. (B) GA monomerization as a function of PC concentration after 21 days of incubation; the GA concentration was 1.0 mg/ml and the injection volume 5 μl. (C) Dependence of monomerization on GA concentration in presence of a fixed lipid concentration of 0.67 mg/ml, recorded after eight days of incubation; the GA concentrations were 0.01, 0.05, 0.1, 0.5, 1.0, and 2.0 mg/ml and the injection volumes were 100, 20, 25, 5, 5, and 2.5 μl, respectively.

in the lipid concentration has two effects on the conformational equilibrium: greater dissociation of the GA dimer and a decrease in the time required to reach equilibrium. Similar results have been obtained for GA concentrations of 0.5, 0.1, and 0.05 mg/ml over the same range of PC:GA molar ratios. It must be emphasized (see below) that both the kinetic and thermodynamic effects in relation to this equilibrium are dependent not only on the absolute PC and GA concentrations but also on the molar ratio of lipid to polypeptide.

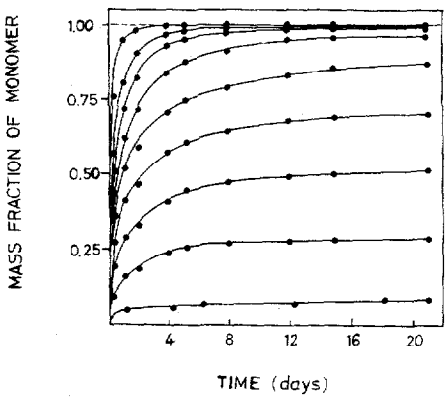

Fig. 5. Kinetic profiles of GA monomerization for a polypeptide concentration of 1.0 mg/ml, at different PC concentrations. The mass fraction of monomer, calculated from the corresponding heights of peaks, is plotted against the time at which each aliquot was taken. Lipid concentration, from bottom to top: 0, 0.33, 0.67, 1.00, 1.30, 1.67, 2.10, 2.50, and 5.00 mg/ml.

TABLE I

VALUES OF PC:GA MOLAR RATIOS, R, AND APPARENT DISSOCIATION CONSTANTS, K_d^{app}, DEDUCED FROM HPLC FOR SEVERAL GA CONCENTRATIONS

GA concentration (mg/ml)							
1.0		*0.5*		*0.1*		*0.05*	
R	K_d^{app} (M)	R	K_d^{app} (M)	R	K_d^{app} (M)	R	K_d^{app} (M)
0	$8.5 \cdot 10^{-6}$	0	$8.8 \cdot 10^{-6}$	0	$8.2 \cdot 10^{-6}$	0	$4.3 \cdot 10^{-6}$
0.79	$1.2 \cdot 10^{-4}$	0.96	$8.7 \cdot 10^{-5}$	0.79	$9.0 \cdot 10^{-6}$	0.72	$4.9 \cdot 10^{-6}$
1.61	$5.7 \cdot 10^{-4}$	1.58	$2.1 \cdot 10^{-4}$	1.61	$2.6 \cdot 10^{-5}$	1.58	$1.3 \cdot 10^{-5}$
2.40	$1.8 \cdot 10^{-3}$	2.40	$7.7 \cdot 10^{-4}$	2.40	$7.2 \cdot 10^{-5}$	2.40	$2.5 \cdot 10^{-5}$
3.12	$6.0 \cdot 10^{-3}$	3.22	$2.6 \cdot 10^{-3}$	4.01	$3.2 \cdot 10^{-4}$	3.22	$4.4 \cdot 10^{-5}$
4.00	$2.9 \cdot 10^{-2}$	4.80	$1.3 \cdot 10^{-2}$	5.04	$7.7 \cdot 10^{-4}$	3.84	$8.3 \cdot 10^{-5}$
5.04	$1.1 \cdot 10^{-1}$			6.48	$2.2 \cdot 10^{-3}$	4.80	$1.6 \cdot 10^{-4}$
				7.99	$1.1 \cdot 10^{-2}$	9.60	$2.6 \cdot 10^{-3}$

Table I summarizes the values of the apparent dissociation constants, K_d^{app}, for the equilibrium in the presence of lipid, estimated from the results at 21 days for different GA concentrations (Fig. 5 and results not shown). In the cases in which the mass fraction of dimer is experimentally measurable the lipid produces an increase in K_d^{app} values of three or four orders of magnitude for each given GA concentration.

In addition to chromatographic analysis, a fluorescence spectroscopy study has been performed, taking advantage of the fluorescent characteristics of the polypeptide Trp residues, in order to clarify the effect of the phospholipid on the GA conformational equilibrium. Fig. 6A shows the emission spectrum of GA for a 0.01-mg/ml sample. Two maxima are observed, at 330 and 338 nm, due to the vibrational structure displayed by the indole ring in a non-polar and aprotic solvent[22]. On addition of PC, two effects are observed in the spectrum: (a) a red-shift up to 342 nm, in the range of lipid compositions assayed, indicating a more polar environment, and (b) an intensification of the overall spectrum, observable at low PC concentrations (up to *ca.* 2 mg/ml), while quenching occurs at higher lipid concentrations. In fact, previous HPLC results[16] have demonstrated a strong binding of PC to GA in tetrahydrofuran, which is expected to cause a static quenching of tryptophan fluorescence.

Since the dimer:monomer ratio varies with the time elapsed after the addition of lipid, a parallel simultaneous kinetic study was designed by using HPLC and spectrofluorometry. Fig. 6B and C shows the results obtained, for a GA concentration of 0.01 mg/ml, expressed as relative fluorescence intensity, I/I_0, (Fig. 6B) and mass fraction of monomer (Fig. 6C) as a function of time (I_0 is the intensity at zero time). From the analysis of these figures the following correlations can be made. The presence of lipid originates in all cases an increase in both fluorescence intensity and mass fraction of monomer. For each lipid concentration, I/I_0 increases as a function of time until a maximum is reached (Fig. 6B). At this moment, simultaneous chromatographic analysis reveals a near-equilibrium situation (Fig. 6C). The position of each maximum depends on the lipid concentration, so that it shifts to shorter times as the PC concentration increases, and the same is observed for the half times in the

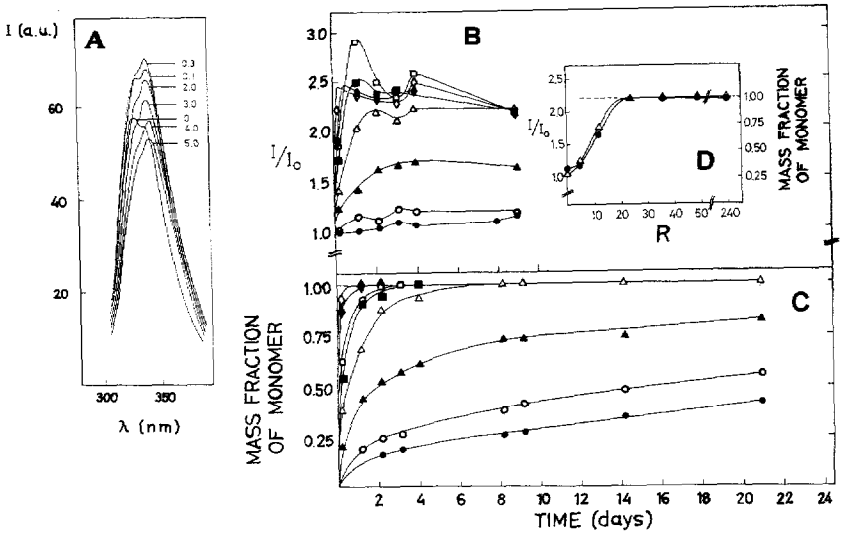

Fig. 6. (A) Effect of addition of PC on the emission spectrum of GA at a concentration of 0.01 mg/ml. The numbers correspond to PC concentrations in the cuvette, expressed in mg/ml. Each emission spectrum was recorded immediately after adding the corresponding lipid solution aliquot and stirring for *ca.* 10 s for complete mixing. (B) Relative fluorescence intensity and (C) HPLC kinetic profiles of the progress of monomerization with a GA concentration of 0.01 mg/ml at different PC concentrations: (●) 0, (○) 0.02, (▲) 0.05, (△) 0.10, (■) 0.15, (□) 0.20, (◆) 0.50, and (◇) 1.0 mg/ml. For each PC concentration, a large sample was prepared and divided into several aliquots to ensure identical concentrations. At each time, two aliquots of the same solution were simultaneously measured by the two techniques. (D) Comparison of results obtained at Day 9 by fluorescence spectroscopy and HPLC. The values of I/I_0 (●) and mass fraction of monomer (△) are plotted *versus* the lipid:polypeptide molar ratio, R (I_0 is the intensity value at zero time in the absence of lipid).

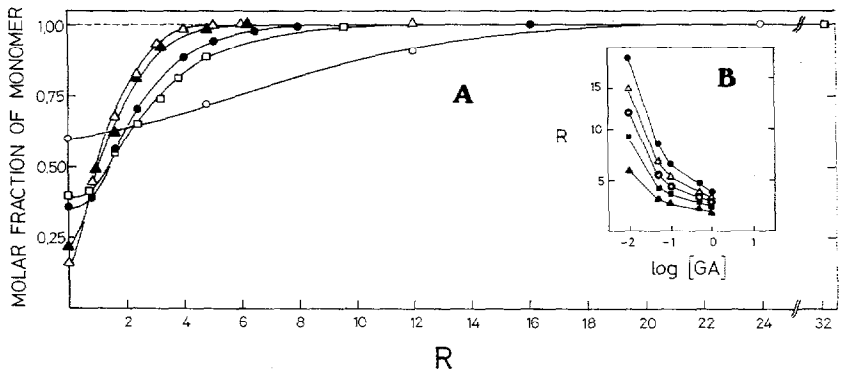

Fig. 7. (A) Effect of phospholipid:polypeptide molar ratio, R, on the GA monomer molar fraction after 21 days (from Fig. 5) at different GA concentrations: (○) 0.01, (□) 0.05, (●) 0.1, (▲) 0.5, and (△) 1.0 mg/ml. (B) Plot of R *versus* log [GA], after 21 days, at different molar fractions of monomer: (●) 0.98, (△) 0.95, (○) 0.91, (■) 0.85, and (▲) 0.75. [GA] is the total GA concentration expressed in mg/ml.

monomerization curves. From this intensity maximum a general quenching phenomenon is observed, where the intensity decreases and stabilizes at longer times when equilibrium is practically achieved. Additional measurements between days 14 and 21 did not reveal significant changes in the fluorescence response with respect to the values at day 9.

It can be assumed that the PC-induced enhancement of fluorescence intensity is mainly due to the appearance of monomeric species, despite the quenching effect of lipids. This assumption is based on (a) the correlation that always exists between the enhancement of observed fluorescence and the displacement of equilibrium towards monomers, and (b) the increase in fluorescence intensity observed for lipid-free GA solutions after monomerization [Fig. 6B and at several other GA concentrations (results not shown)]. The quenching effect of the lipid also seems to increase when the equilibrium is approached. This could be a consequence of a progressive, tighter rearrangement of the lipid around the GA monomer. On the other hand, additional experiments (results not shown) have revealed that addition of water to GA samples in tetrahydrofuran causes a higher percentage of monomeric forms and an overall enhancement of the fluorescence intensity similar to that observed by addition of lipid.

From the results in Fig. 6B and C both parameters, I/I_0 and mass fraction of monomer, at day 9, have been plotted *versus* the PC:GA molar ratio, R (Fig. 6D). It can be seen that the results of both techniques are in very good agreement and that, for a PC concentration of 0.1 mg/ml ($R = 24$), both curves reach a plateau corresponding to 100% of fully lipid-bound monomers.

In Fig. 7A the results are presented in an alternative form. The molar fraction of monomer after 21 days is plotted *versus* R for each GA concentration (data from Figs. 5, 6C, and results not shown at three other GA concentrations). For a given R value, the extent of monomerization depends on the initial GA concentration, so the curves shift to a higher molar fraction of monomer with increasing GA concentration. The curve for GA at 0.01 mg/ml has been included, but some deviation from the general behaviour is observed. This is probably due to the fact that, at this time, GA conformational equilibrium is not completely achieved (Fig. 6C) and, therefore, the monomer concentration is lower than expected.

It is noteworthy that for a GA concentration of 0.1 mg/ml, only three molecules of PC per molecule of GA are able to stabilize *ca.* 94% of monomer. Since the ratio of lipid molecules necessary to obtain a given extent of monomerization decreases with increasing GA concentration, the R values have been plotted *versus* log [GA] at different molar fractions of monomer (Fig. 7B) in order to estimate, for high polypeptide concentrations, a theoretical minimum number of lipid molecules that can stabilize GA as a monomer. This plot seems to indicate that, when the GA concentration increases, all the curves apparently tend to an approximate R value of 1–2 molecules of PC per molecule of GA monomer. This would mean that between one and two molecules of phospholipid could induce total peptide monomerization.

DISCUSSION

Based on previous results from spectroscopic studies[8], it has been shown that the conformations of GA in dioxane are $\uparrow\downarrow \pi\pi_{LD}$ double helices for the dimer and

a π_{LD} helix for the monomer. Consequently, in tetrahydrofuran, a less polar solvent than dioxane, similar helical conformations for GA dimeric and monomeric forms are to be expected. In fact, the elution volumes obtained for each GA species are larger than expected, and this supports the argument for a compact structure for both dimer and monomer. In this connection, it is known that a change in conformation of a macromolecule generally results in a change in its refractive index[23]. We have observed that the peak ratios in the refractive index response do not coincide with the UV response. This can be attributed to the fact that each helix type has a different refractive index due to its different conformation, if we assume, as mentioned in Results, that the molar extinction coefficient is the same for dimer and monomer Trp residues.

In tetrahydrofuran, the monomerization process of GA is slow, in agreement with spectroscopic results from studies in non-polar solvents[8], whereas it is more rapid in polar solvents as deduced from Fig. 2 and previous papers[9,10]. The presence of phospholipid accelerates the dissociation of dimer, so that the higher the concentration of PC, the higher the rate of monomerization. In this connection, Sychev *et al.*[8] have described a striking increase (by six orders of magnitude) of the dimer dissociation rate in liposomes as compared with a non-polar solvent.

On addition of PC, two events take place: (a) PC binds to both dimer and monomer, the latter being more solvated by lipid than the dimer, as deduced from chromatographic analysis of vacant peaks[17], and (b) the dimer–monomer equilibrium is more displaced towards the monomer; this effect becomes more marked as the PC concentration increases. In this connection, the shift to higher wavelengths observed in fluorescence emission spectra (Fig. 6A) is attributable to the location of lipid polar head groups in the vicinity of Trp residues. This assumption is also supported by previous chromatographic results showing that PC–GA interactions in tetrahydrofuran occur with a simultaneous release of some of the solvation water originally bound to the lipid polar head[17]. This observation is in line with a recently proposed model for protein–lipid interactions in membranes containing Ca-ATPase, in which some of the phospholipid head groups of the membrane interact directly with the protein[24].

In tetrahydrofuran, the effect of the addition of water on the established GA equilibrium in the presence of lipid must be considered. When a few microlitres of water are added to a sample containing lipid-solvated monomer, chromatographic analysis reveals some reversion of the equilibrium towards dimeric forms, accompanied by a decrease in fluorescence intensity. A plausible explanation for this phenomenon is that water molecules again solvate some lipid polar heads, preventing them from interacting with GA monomer. Therefore, some reassociation occurs, leading to a higher proportion of dimeric species.

The fluorescence enhancement observed after the addition of lipid (Fig. 6A and B) could arise from two independent, non-exclusive phenomena, either a direct interaction between the bound lipid and Trp residues, or an indirect interaction through a dimer–monomer conformational change. Although not excluded, the first alternative does not seem to be the determinant factor, because experiments based on the addition of PC to N-acetyl-L-tryptophanamide (NATA) in tetrahydrofuran, in the same concentration range as GA, have shown either quenching or very little enhancement of NATA fluorescence. The most likely explanation is that GA fluo-

rophores are following a lipid-induced conformational change, since we have observed that in tetrahydrofuran the fluorescence intensity of the monomer is higher than that corresponding to the dimer (*e.g.*, Fig. 6B). This behaviour is different from that observed in ethanol, where GA monomerization is accompanied by a decrease in fluorescence emission intensity, in agreement with previous data from Veatch *et al.*[10]. The difference in the fluorescence intensity ratio of dimeric and monomeric species in ethanol relative to tetrahydrofuran can be interpreted according to the different degree of secondary structure of the GA monomer in both solvents. The higher intensity of the monomer relative to the dimer, found in tetrahydrofuran, would support the assumption that the GA monomer is highly folded in solvents of very low polarity[8,11,14]. The strong lipid-induced enhancement of GA fluorescence intensity (nearly three-fold at the maximum, Fig. 6B) would suggest that in the presence of lipid, the GA conformational equilibrium is not only displaced towards monomeric forms, but the lipid-solvated monomer also maintains an ordered conformational structure.

The shape of the curves in Fig. 7A seems to indicate a sigmoidal behaviour, which suggests co-operativity. The low value of R (1 or 2) apparently required for complete monomerization at very high GA concentrations (Fig. 7B) would support the idea that the initial binding of some lipid molecules to the dimer causes its dissociation and the appearance of additional, higher-affinity binding sites on the monomer.

As a conclusion, a simplified multiple equilibrium model for GA–PC interactions in tetrahydrofuran can be proposed (see Fig. 8), to account for all the experimental observations reported here.

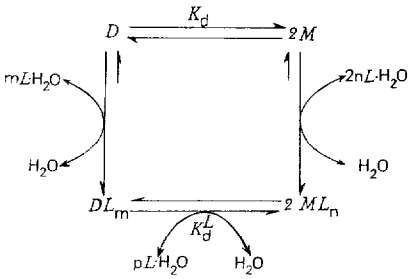

Fig. 8. Simplified multiple equilibrium model for GA–PC interactions in tetrahydrofuran. D = dimer, M = monomer, $L \cdot H_2O$ = hydrated lipid, K_d^L = global dissociation constant in the presence of lipid, m = 1, 2, 3, ...; n = 1, 2, 3, ...; p = 1, 2, 3, ...; with 2 n > m which means that two molecules of monomer are more solvated by lipid than one molecule of dimer, as deduced from previous work[17].

It should be mentioned that the good resolution achieved with this column for the two conformational species of GA (both of low molecular weight) has allowed us to visualize directly the dimer–monomer conformational equilibrium of this polypeptide in non-polar solvents in the presence and absence of phospholipid. Size-exclusion HPLC has proved extremely useful for quantifying the actual composition of the system at each moment. The simultaneous use of another technique,

such as spectrofluorometry, provides an additional approach to the complete system. The correlation between the results obtained by both techniques allows a better interpretation of the molecular associations.

This methodology offers a chance to investigate more complex hydrophobic proteins, *e.g.* proteolipids, since as a consequence of their chemical nature, their chromatographic behaviour is somewhat unusual, depending on association with lipids or on their state of aggregation[25].

ACKNOWLEDGEMENT

We thank Dr. L. Franco for helpful discussions and critical reading of the manuscript.

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