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CONFORMATIONAL SPECIES OF GRAMICIDIN A IN NON-POLAR SOLVENT

A KINETIC AND THERMODYNAMIC TREATMENT IN THE ABSENCE AND PRESENCE OF PHOSPHATIDYLCHOLINE AS STUDIED BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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A kinetic and thermodynamic study has been carried out to characterize quantitatively the conformational equilibrium of gramicidin A (GA) in tetrahydrofuran at different peptide concentrations in the absence and presence of egg yolk phosphatidylcholine by using size-exclusion high-performance liquid chromatographic analysis. In the absence of lipid, the experimental data fit a simple dimer-monomer equilibrium, the rate and equilibrium constants for the dissociation process being $(1.6\pm0.7)\times10^{-7}$ s^{-1} and $(8.5\pm0.3)\times10^{-6}$ M, respectively. A higher extent of monomerization and a decrease in the time required for reaching equilibrium are detected in the presence of phospholipid, the kinetic and thermodynamic effects depending on both lipid and GA concentrations. In order to account for these observations a cyclic equilibrium mechanism is proposed which is analysed in terms of four conformational species, namely, free monomer, free dimer, lipid-bound monomer and lipid-bound dimer. The results obtained are discussed in relation to recent literature data on lipid-protein interactions.

1. Introduction

In the last few years, the linear DL-alternating pentadecapeptide gramicidin A (GA) has been widely used in studies of lipid-protein interactions, as a model for the hydrophobic part of intrinsic membrane proteins [1–4]. On the other hand, it has been clearly demonstrated that, in the membrane as well as in organic solvent, GA undergoes dimer-monomer conformational transitions [5–8].

Thus, extensive attention has been directed to the study in organic solvents of the conformational features of linear peptides formed by regularly alternating D and L residues, as synthetic models of gramicidins. The nature and relative stability of monomeric and dimeric species of DL-oligovalines in cyclohexane, carbon tetrachloride or chloroform solutions have been investigated by spectroscopic techniques [9–11]. Different slowly interconverting species are present in these solvents and at least one of them is a double-stranded helical dimer. With oligophenylalanine, NMR data indicate that in chloroform solution dimeric and tetrameric species in rapid equilibrium exist, which are double-stranded helical dimers and the head-to-head dimerization product of the parallel one, respectively [12].

Recently, it has been reported that gramicidin exerts a dramatic influence on lipid-phase behaviour in model membrane systems, inducing

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bilayer to non-bilayer transitions [13–16]. When GA is incorporated into phosphatidylcholine (PC) or phosphatidylethanolamine (PE) model membranes, a head group dehydration of the lipid molecules occurs and a reversed hexagonal H_{II} phase is induced, as deduced from differential scanning calorimetry and NMR techniques [15,16]. Apparently, GA molecules prefer to organize in the H_{II} phase as monomeric tubular structures, in which the polypeptide decreases the molar order and increases the rate of motion of the phosphate moiety [16]. For dioleoyl-PC, it has been shown by means of small-angle X-ray diffraction and NMR studies that in excess water this H_{II} phase is very rich in GA, with a molar ratio of peptide to lipid of about 1 : 2.5 [17].

Recently, we have demonstrated the usefulness of size-exclusion high-performance liquid chromatography (HPLC) to visualize directly the time course of the dimer-monomer conformational equilibrium of GA in a non-polar solvent [18]. In order to obtain a better molecular understanding of the way in which PC is able to induce changes in the GA conformational equilibrium, a systematic kinetic and thermodynamic study has been carried out in the present paper at different GA concentrations in the absence and presence of the phospholipid. By combining the results obtained from this technique and information derived from previous chromatographic and spectrofluorometric studies [18,19], we propose a cyclic equilibrium mechanism to explain the changes observed in the presence of lipid, which is analysed in terms of the four individual conformational species present at equilibrium. The results obtained are related to literature data on GA incorporated into membranes [13–17] and on other synthetic polypeptides in organic solution [9–12].

Taking into account that the molecular structure of the conductive transmembrane channel formed by GA is at present strongly debated [20–24], size-exclusion HPLC is revealed as potentially useful for studying the molecular organization of the peptide in the membrane, by performing chromatographic analysis of gramicidin incorporated into liposomes.

2. Experimental

2.1. Materials

GA was supplied by Koch Light Labs. and was used without further purification. Egg yolk PC was purchased from Merck and purified according to the column chromatographic method of Singleton et al. [25]. The phospholipid gave a single spot when analysed by thin-layer chromatography. Tetrahydrofuran (THF) was spectroscopic grade (Merck). It was passed through a 0.45 μm Micro Filtration Systems regenerated cellulose filter before use.

2.2. Methods

The liquid chromatograph consisted of an M-45 solvent-delivery system and a U6K universal injector from Waters Associates. The detector used was a Varian Varichrom variable-wavelength ultraviolet-visible detector. The system was equipped with a 100 nm pore-size Ultrastyragel column (30 × 0.78 cm inner diameter) from Waters. The chromatograms were recorded by using a Yokogawa Electric Works dual-channel recorder.

Stock GA solutions were prepared by dissolving the polypeptide in THF at concentrations of 1.0, 0.5, 0.1 and 0.05 mg/ml. Aliquots up to 100 μl from a highly concentrated stock PC in THF solution (300 mg/ml) were immediately added in all cases to 10 ml of the corresponding GA solution and the samples were stirred for 1 min for complete mixing. For the kinetics in the presence of PC, zero time was taken immediately after the addition of the phospholipid. 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 THF at a flow rate of 1.0 ml/min. The injection volume was variable so that the absolute amount of injected GA for all the assayed concentrations was similar: 5, 10, 25 and 20 μl were injected for GA concentrations of 1.0, 0.5, 0.1 and 0.05 mg/ml, respectively.

3. Results

We have recently reported the separation of two conformational species of GA in THF, identified as dimeric and monomeric forms [18]. These conformational species interconvert so slowly at room temperature that reaching equilibrium may require several days. The presence of PC drastically alters this equilibrium. Typical chromatograms of GA at 1.0 mg/ml in lipid-containing THF solutions are shown as an example in fig. 1 as a function of time. The first eluting peak corresponds to the dimer and the second one to the monomer. It is evident that the elapsed time results in a displacement of equilibrium from GA dimer (zero time) towards monomeric forms.

Some considerations can be made on the mechanisms involved in the elution process. First of all, we have previously verified that PC strongly interacts with the polystyrene-divinylbenzene reticular matrix of the gel [19,26]. As a consequence, PC is probably no longer associated with either the dimer or the monomer during elution, so that the emerging peaks observed in the chromatograms correspond to lipid-free dimeric and monomeric species. This observation is supported by: (a) the elution volumes (and the shape) of the peaks from lipid-containing samples are identical to those obtained with lipid-free ones; (b) phosphorus analysis of eluted fractions corresponding to both peaks and to baseline did not reveal significant differences. Therefore, each peak observed in the chromatograms corresponds to total free dimeric or monomeric species, derived from the sum of free + lipid-bound forms existing before injection. The formation of PC-bound dimeric and monomeric complexes in THF has been also demonstrated in previous papers [19,26].

Furthermore, after the dissociation of the phospholipid from the polypeptide due to binding to the support, it is not likely that the ratio of free species is altered through the column, since the conformational equilibrium of GA in THF is extremely slow (several days [18]) and the time required for the analysis is only 9 min.

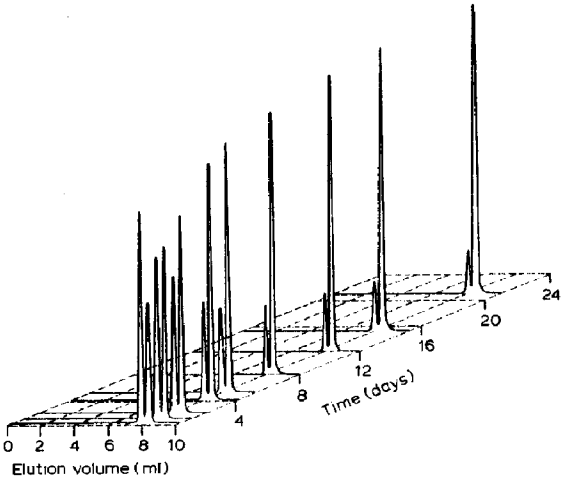


Fig. 1. Elution profiles of GA at a fixed PC concentration of 1.3 mg/ml, as a function of incubation time. GA concentration was 1.0 mg/ml. Samples were monitored by absorbance at 294 nm. The injection volume was 5 μl in all cases. In the chromatograms, the peak eluting at 7.9 ml corresponds to the GA dimer and the peak at 8.4 ml to the GA monomer.

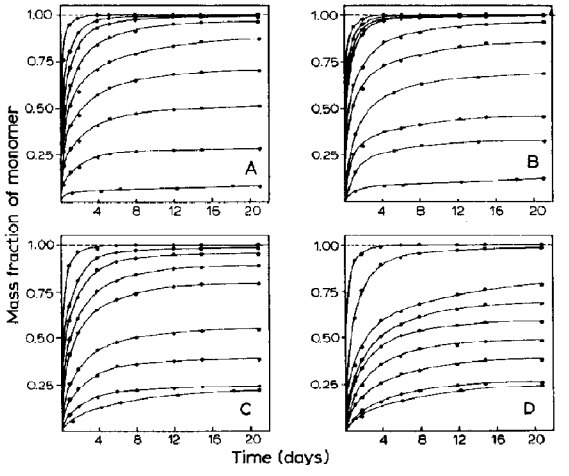


Fig. 2. Kinetic profiles of GA monomerization for four polypeptide concentrations at different PC concentrations. The mass fraction of monomer, calculated from the heights of peaks (fig. 1, as an example), is plotted against the time at which each aliquot was taken. (A) GA concentration: 1.0 mg/ml. Lipid 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. (B) GA concentration: 0.5 mg/ml. Lipid concentration, from bottom to top: 0, 0.20, 0.33, 0.50, 0.67, 1.00, 1.30, 1.50, 1.67 and 2.50 mg/ml. (C) GA concentration: 0.1 mg/ml. Lipid concentration, from bottom to top: 0, 0.033, 0.067, 0.10, 0.17, 0.21, 0.27, 0.33 and 0.67 mg/ml. (D) GA concentration: 0.05 mg/ml. Lipid concentration, from bottom to top: 0, 0.015, 0.033, 0.050, 0.067, 0.080, 0.10, 0.20 and 0.67 mg/ml.

The kinetic results for GA monomerization in the absence and presence of PC at GA concentrations of 1.0, 0.5, 0.1 and 0.05 mg/ml are presented in fig. 2. We have verified (results not shown) that the GA free dimeric and monomeric species have essentially the same molar extinction coefficient in THF, by following monomerization kinetics by absorbance measurements at 294 nm. Thus, since the dimer and monomer peaks are very symmetrical (fig. 1), it was possible to evaluate the mass fraction of each species directly from the peak heights in the chromatograms.

In the absence of lipid, a detailed kinetic analysis has been carried out, based on a simple dimer-monomer equilibrium, and the rate constant for the dissociation process has been determined.

Let us assume the following equilibrium for gramicidin:

$$M_2 \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} 2M \tag{1}$$

$$\frac{dx}{dt} = k_1([M_2]_0 - x) - k_{-1}4x^2 \tag{2}$$

The concentrations at equilibrium are independent of time, so

$$K_d = \frac{4x_e^2}{[M_2]_0 - x_e} = \frac{k_1}{k_{-1}} \tag{3}$$

where K_d is the dissociation equilibrium constant (M). $[M_2]_0$ denotes the dimer concentration at zero time; x refers to the concentration of dissociated dimer at a given time, t, and x_e to that of dissociated dimer at equilibrium and k_1 (s^{-1}) is the rate constant of the direct process.

After substitution for k_{-1} from eq. 3 into eq. 2 and rearrangement, the following integrated equation is obtained:

$$\ln\frac{x_e - x}{\dfrac{[M_2]_0 x_e}{[M_2]_0 - x_e} + x} = \ln\frac{[M_2]_0 - x_e}{[M_2]_0} - \frac{2[M_2]_0 - x_e}{x_e}k_1 t \tag{4}$$

If the dimer concentration at equilibrium is $[M_2]_e = [M_2]_0 - x_e$, then $x_e = [M_2]_0 - [M_2]_e$, and $x = [M_2]_0 - [M_2]$, so that eq. 4 can be rewritten as:

$$\ln\frac{[M_2] - [M_2]_e}{\dfrac{[M_2]_0}{[M_2]_e}([M_2]_0 - [M_2]_e) + ([M_2]_0 - [M_2])} = \ln\frac{[M_2]_e}{[M_2]_0} - \frac{[M_2]_0 + [M_2]_e}{[M_2]_0 - [M_2]_e}k_1 t \tag{5}$$

The data in the absence of lipid in fig. 2 (bottom curves), are plotted in fig. 3 according to eq. 5. In order not to overcrowd the figure, only two GA concentrations are included. For simplicity, the whole fraction of the first member in eq. 5 will be referred to as P. A good fitting is observed in all cases, which demonstrates, on the one hand, the validity of the model in eq. 1, and on the other, the accuracy in the chromatographic determination of dimer and monomer concentrations. From the slope of each straight line, k_1 values of 1.1×10^{-7}, 1.1×10^{-7}, 2.5×10^{-7} and 3.5×10^{-7} s^{-1} have been obtained for GA concentrations of 1.0, 0.5, 0.1 and 0.05 mg/ml, respectively. Since the kinetics in fig. 2D has probably not reached equilibrium, it is more adequate to make use of the data in fig. 2A–C in order to determine an average value for both the monomerization rate constant and the dissociation equilibrium constant. Thus, $k_1 = (1.6 \pm 0.7) \times 10^{-7}\,s^{-1}$ and $K_d = (8.5 \pm 0.3) \times 10^{-6}$ M have been obtained. These values, calculated in THF for the first time, fit very well the sequence reported by Veatch and Blout [5] from spectroscopic measure-

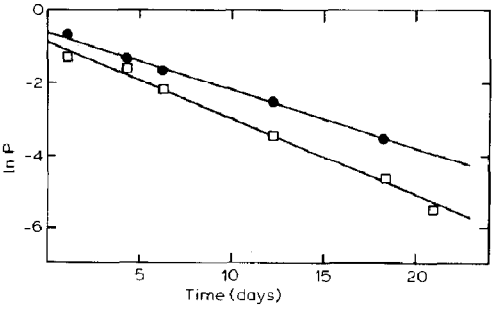
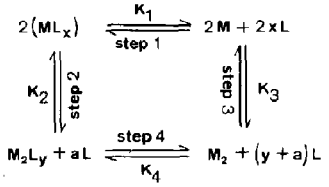


Fig. 3. Plot of ln P vs. time according to eq. 5, for GA concentrations of 1.0 mg/ml (□) and 0.1 mg/ml (●).

ments for the rate and equilibrium constants as a function of solvent polarity.

Concerning the presence of PC, an increase in lipid concentration has, for all the GA concentrations assayed, two effects on the conformational equilibrium: a higher extent of monomerization and a decrease in the time required for reaching the equilibrium. The kinetic and thermodynamic effects are dependent on the absolute PC or GA concentrations as well as on the lipid-polypeptide molar ratio (fig. 2A–D).

Let us now consider the possible mechanism for GA monomerization in THF induced by the phospholipid. In the light of our present information on these systems, namely, (i) the simplified multiple model proposed previously by us for GA-PC interactions [18]; (ii) the observation that both dimeric and monomeric species of GA bind to the lipid [19,26]; and (iii) the fact that two monomeric species are more solvated by lipid than a dimeric one [19], the following mechanism can be proposed:



In this scheme, x and y account for the lipid molecules bound to the monomeric and dimeric species, respectively, a referring to the additional lipid molecules bound to the monomer relative to the dimer. The lipid, L, can bind to either M_2 or M forms, the equilibrium constants being:

$$K_1 = \frac{[M][L]^x}{[ML_x]} \tag{6}$$

$$K_2 = \frac{[ML_x]^2}{[M_2L_y][L]^a} \tag{7}$$

$$K_3 = \frac{[M]^2[L]^{2x}}{[M_2][L]^{y+a}} \tag{8}$$

$$K_4 = \frac{[M_2][L]^{y+a}}{[M_2L_y][L]^a} \tag{9}$$

and K_d^{app} the total effective equilibrium dissociation constant:

$$K_d^{app} = \frac{([M] + [ML_x])^2}{([M_2] + [M_2L_y])} \tag{10}$$

Rearrangement of eq. 10 taking into account eqs. 6, 8 and 9 gives:

$$K_d^{app} = K_3 \frac{[1 + [L]^x/K_1]^2}{1 + [L]^y/K_4} \tag{11}$$

since $2x = y + a$. This equation is similar to that proposed by Quay and Condie [27] to explain the self-association of melittin under conditions of different pH and ionic strength.

Let us assume that the monomer or dimer peaks appearing in the chromatograms are due to the sum of free and lipid-bound species in each case. The data at equilibrium (21 days) in the presence of lipid in fig. 2A–D can be used to determine the total concentration of monomeric, $[M]_T$, and dimeric, $[M_2]_T$, species at each lipid concentration and to obtain K_d^{app} by means of eq. 10.

Since (i) K_3 corresponds to the dissociation constant of GA in the absence of lipid, $K_3 = 8.5 \times 10^{-6}$ M, (ii) based on previous studies of binding of PC to dimeric GA, the dissociation constant K_4 is about 2×10^{-5} M [19], (iii) the lowest number of bound lipid molecules needed for complete monomerization of GA has been estimated as about 5 ($x = 5$) [18] and (iv) K_d^{app} is known from chromatographic data through eq. 10, an estimation of K_1 can be obtained from eq. 11. For this purpose, free lipid concentration, [L], has been calculated using total GA and PC concentrations of 1.0 and 2.1 mg/ml, respectively, and the stoichiometry $x = 5$ for the lipid-bound monomeric species, ML_x. Thus, $[L] = 3.2 \times 10^{-5}$ M has been obtained. In addition, an estimation of the y value is also required for the determination of K_1. Since two monomers are more solvated by lipid than one dimer [19], values for y lower than 10 have been checked. For $1 < y < 10$, K_1 remains unalterable, whereas for $y < 1$, a very slight variation in K_1 value is observed (one order of magnitude). From an iterative process involving recalcu-

lation of x and y by using eqs. 6, 7 and 9, the most consistent values obtained are: $x = 5$, $y = 1.0$, $K_1 = 2 \times 10^{-26}$ and therefore, $a = 9.0$.

By using K_1, K_3 and K_4 values and taking into account that the law of microscopic reversibility leads to the relationship $K_1^2 K_2 = K_3 K_4$ for the cyclic mechanism proposed, the resulting value for K_2 is about 10^{41}.

Since the conformational equilibrium of GA is sensitive to both lipid and GA concentrations and four species are possible, namely, free dimer, free monomer, lipid-bound dimer and lipid-bound monomer, it is interesting to consider the variation of each one of these species at equilibrium, at different GA concentrations, as a function of lipid concentration. Taking into account that

$$[M]_T = [M] + [ML_x] \quad (12)$$

and

$$[M_2]_T = [M_2] + [M_2L_y] \quad (13)$$

and using eqs. 7 and 8, the following second-grade equation is obtained:

$$\left(\frac{1}{K_2} + \frac{1}{K_3}\right)[ML_x]^2 - \frac{1}{K_3} 2[M]_T[ML_x] + \frac{1}{K_3}[M]_T^2 - [M_2]_T = 0 \quad (14)$$

which allows one to determine $[ML_x]$ as a function of lipid concentration once the experimental data K_3, $[M]_T$ and $[M_2]_T$ are known. It should be noted that $1/K_2$ is negligible with respect to $1/K_3$. The evaluation of $[ML_x]$ is assumed to be very accurate since the three coefficients in eq. 14 have been obtained directly from chromatographic results. [M] is then derived from eq. 12, $[M_2]$ from eq. 8 and $[M_2L_y]$ from eq. 13.

Fig. 4A–D shows the results obtained for the four GA concentrations under study as a function of PC concentration. The following considerations on the changes observed in the individual species can be made: (i) ML_x is in all cases the predominant species at equilibrium at high lipid concentration, whereas M_2 predominates at low lipid concentration; (ii) [M], which is lower than $[M_2]$ at low lipid concentration, increases relative to the dimer at high lipid concentrations, this effect being more pronounced as GA concentration decreases (fig. 4D); (iii) a slight increase in $[M_2L_y]$ is observed, except for the lowest GA concentration, where it is practically negligible.

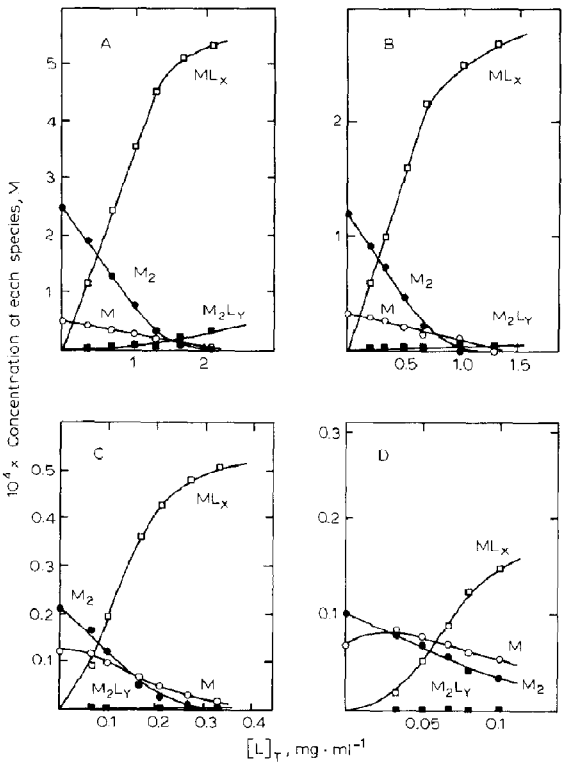


Fig. 4. Dependence of the concentration of each one of the gramicidin species at equilibrium on total PC concentration, $[L]_T$. GA concentrations: (A) 1.0 mg/ml; (B) 0.5 mg/ml; (C) 0.1 mg/ml and (D) 0.05 mg/ml. (●) Free dimer, (■) lipid-bound dimer, (○) free monomer and (□) lipid-bound monomer.

An alternative plot of the results in fig. 4 as a function of GA concentration at different PC/GA molar ratios, R (not shown), would show that at low R (< 2) the predominant species is always M_2 whereas for $R > 2$ a dramatic increase in lipid-bound monomer occurs, this species being derived basically from the free dimeric one.

Finally, an analysis of the kinetic data in the presence of PC has been carried out in order to clarify the mechanism of lipid-induced dissociation of the dimer. The data in fig. 2A at different PC concentrations have been plotted in fig. 5 according to eq. 5, replacing free dimer and mono-

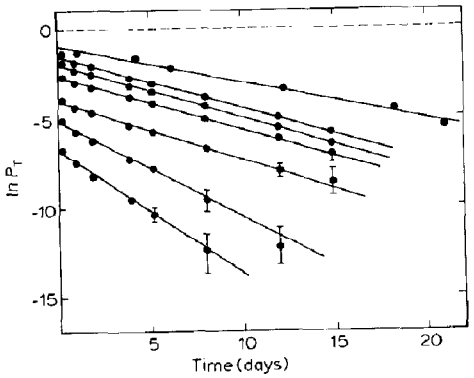


Fig. 5. Plot of ln P_T vs. time according to eq. 5, modified for total dimeric and monomeric species as described in the text, for different lipid concentrations. GA concentration was 1.0 mg/ml. PC concentrations were, from top to bottom (in mg/ml): 0, 0.67, 1.00, 1.30, 1.67, 2.10 and 2.50.

mer concentrations for the total (free + lipid-bound) ones, these latter deduced directly from the chromatographic measurements. The figure plots ln P_T as a function of time, subscript T referring to total (free + lipid-bound) species. Note that the values of ln P_T near equilibrium at high monomer concentrations are affected by a high error and they should not be taken into account. It can be seen that the fitting is good in all cases, which suggests that the reaction rate in the presence of PC is governed by the sum of two slow steps:

$$\text{(i)}\ M_2 \overset{\text{slow}}{\rightleftharpoons} 2M \qquad \text{(step 3)}$$

$$\text{(ii)}\ M_2L_y \overset{\text{slow}}{\rightleftharpoons} 2ML_{y/2} \qquad \text{(step 2a)}$$

the binding of the additional PC molecules to the emerging monomer being very fast:

$$\text{(iii)}\ 2ML_{y/2} + aL \overset{\text{fast}}{\rightarrow} 2ML_x \qquad \text{(step 2b)}$$

The increased lipid-induced monomerization rate is similar to that observed for more polar solvents [5] or for THF/water mixtures (results not shown). This would suggest that the binding of PC to GA dimer causes a catalytic-like effect, probably due to a polar microenvironment of lipid heads which somehow would unstabilize the dimer intermolecular hydrogen bonds (step 2a).

4. Discussion

As expected from spectroscopic data of GA in dioxane [5,8,28], the conformational equilibrium of GA in THF is very slow, 21 days or more, depending on the GA concentration used (fig. 2).

The chromatographic data at equilibrium in the absence of lipid (bottom curves in fig. 2A–D) allow one to determine an average dissociation constant for GA, $K_d = 8.5 \times 10^{-6}$ M, without the uncertainty reported for other techniques [5,27].

In the absence of lipid, the chromatograms of GA at high polypeptide concentration injected immediately after dissolving show only one peak which probably corresponds to a $\uparrow\downarrow\pi\pi_{LD}$ antiparallel double-helical dimer stabilized by 28 intermolecular hydrogen bonds [29]. Based on the demonstrated conformational heterogeneity of the gramicidin isolated species in non-polar solvents [28,29], parallel double-helical dimers or double helices of opposite handedness could not be totally excluded. On the other hand, a very small percentage of $\vec{\pi}_{LD}\vec{\pi}_{LD}$ head-to-head dimeric forms cannot be excluded in THF, in a similar way to that reported from infrared studies in dioxane by Sychev et al. [28]. However, since we have observed no additional peak in the chromatograms, it is likely that such a dimer stabilized by only six intermolecular hydrogen bonds would dissociate through the chromatographic column, so that it would emerge as a monomer.

The data in fig. 2 indicate that the conformational equilibrium of GA is highly sensitive to the presence of PC. Based on our present knowledge of GA-PC systems in THF, a more complete equilibrium mechanism is proposed and developed in this paper, which corroborates the previous qualitative approach [18]. In addition, the chromatographic results obtained at equilibrium have been used to estimate not only the phospholipid dependence of the dissociation constant, K_d^{app}, but also K_1 and K_2 equilibrium constants in the scheme and the concentration of each free and lipid-bound dimeric and monomeric species. The values obtained for K_1 and K_2 clearly indicate that the PC exhibits a very high affinity for the monomer.

On the other hand, K_d^{app} values (summarized

in ref. 18) are always higher than those in the absence of lipid, increasing up to five orders of magnitude relative to K_d (or K_3) ones, a change similar to that observed for GA in dimethyl sulfoxide relative to dioxane [5]. The effect of the phospholipid on the conformational equilibrium is markedly higher (several orders of magnitude) than that caused by water added to the solution, when the lipid/peptide and water/peptide mole ratios are taken into account (results not shown). As it will be discussed below, structural features of the PC, especially of the polar head moiety, seem to be determinant for the observed changes.

In general, peptide-lipid contacts are extensive and peptide-peptide interactions relatively rare, except at low R values (< 2) for the higher GA concentrations, where the free dimeric GA structure predominates. The fact that, in the presence of increasing concentrations of phospholipid, the dimer-monomer transition is affected in a different manner at each GA concentration suggests that the initial free dimer/free monomer ratio can be essential for interaction with the lipid. An interesting observation from the plot in fig. 4C and D is the delay in the rise of ML_x species with a shape typical of a cooperative process.

It is evident (fig. 4) that in the organic solvent peptide-lipid interactions maintain a monomer conformation rather than a double-stranded helix. Although the GA conformation in THF in the presence of phospholipid may not be equivalent to the structure in membranes, it has been suggested from CD studies in vesicles that peptide-lipid interactions are important for maintaining the gramicidin head-to-head dimeric channel structure [30], which is formed by the juxtaposition of two monomeric forms.

In this connection, it has been recently reported that gramicidin, when incorporated into PC bilayers at a peptide-to-lipid molar ratio of 1:20 or higher, forms large aggregates which are able to induce the reversed hexagonal phase H_{II} [13,15], where it is suggested that GA in monomeric form interacts with the phospholipid [16].

Some other considerations can be made in relation to GA-PC interactions in THF. It has been verified that the interaction causes a release of the solvation water of the phospholipid polar head [19]. In contrast, the addition of either different chain length *n*-alkanes or triacylglycerols (result not shown) does not alter apparently the conformational equilibrium. Thus, the main binding portion can be ascribed to the phosphate moiety of the PC. A double-stranded structure such as that adopted by GA in non-polar solvents, which is very stable and exhibits a lifetime measured in days in THF, can decrease its lifetime up to hours or even minutes (depending on lipid concentration), basically as a consequence of the interaction of the polypeptide with the lipid polar heads.

In addition, it has been suggested, from DSC and NMR studies of GA-induced H_{II} phase, that the head group of the phospholipid plays a determinant role in its interaction with GA [16] and that the peptide affects the hydration properties of lipids, causing a dehydration of the head groups, probably through a direct interaction [15].

Let us now consider the possible monomer organization. In the absence of lipid, it seems very likely that the monomeric species in THF could be a single-stranded π-helix similar to that observed either for the monomer of GA and analogs in dioxane [28] or for the monomer of the DL-oligovalines in chloroform [9–11]. On the other hand, we have observed that GA monomerization in THF is accompanied by a slight enhancement of the intensity of fluorescence emission, which is in all cases lower than that observed for the lipid-induced monomerization [18,19]. Since it has been also verified that the addition of PC to a THF solution of the fluorophore model *N*-acetyl-L-tryptophanamide gives rise to no fluorescence enhancement [18], it can be suggested that the lipid-induced fluorescence changes are probably related much more to a change in the structure of the lipid-solvated monomer helix, which would become somehow more compact or rigid, than to a change in the fluorophore environment. We are at present performing additional fluorescence and fluorescence polarization experiments in solvents of different polarity in the absence and presence of PC which seem to support the above assumption. In this connection, such an interpretation seems plausible, based on the widely reported evidence that binding of phospholipids to polypeptide chains in aqueous solution (e.g., from

lipoproteins [31], hormones [32] or histone H1 [33]) causes anisotropic changes [31] or an increase in the relative quantum yield of the fluorophores [33] accompanied by changes in the CD spectrum [32,33], consistent with an increase in the α-helix content.

In summary, this study shows that size-exclusion HPLC analysis allows one to characterize quantitatively the dimer-monomer conformational equilibrium of GA in a non-polar solvent. The advantages in evaluating dissociation constants, dissociation rate constants and even the concentration of each species at equilibrium in the absence or presence of phosphatidylcholine are demonstrated. In addition, it should be emphasized that such a methodology can provide a new approach to the study of the molecular organization of gramicidin in the membrane, which is currently a matter of controversy [20–24]. Chromatographic studies of gramicidin incorporated in liposomes are in progress.

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