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NEW HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY-BASED METHODOLOGY FOR MONITORING THE CONFORMATIONAL TRANSITIONS OF SELF-ASSOCIATING HYDROPHOBIC PEPTIDES, INCORPORATED INTO LIPOSOMES

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SUMMARY

A new high-performance size-exclusion chromatographic strategy is reported for the analysis of the hydrophobic self-associating peptide gramicidin A, incorporated into artificial phospholipid vesicles (liposomes). The method is based on the direct injection of a few microlitres of the gramicidin A-containing liposome suspension into the column, which is eluted with a non-polar solvent, such as tetrahydrofuran. The type and amount of information which can be derived from this methodology have been evaluated. Using this chromatographic approach, a correlation has been unambiguously shown to exist between the organization of the peptide in the vesicles and a number of variables involved in the method of preparation of liposomes. Finally, a gramicidin A conformational transition has been monitored in the phospholipid vesicles which proved to be dependent on the class of phospholipid present in the liposome.

INTRODUCTION

In previous papers we have described the high-performance size-exclusion chromatographic (HPSEC) characterization of the dimer–monomer conformational equilibrium of the hydrophobic peptide gramicidin A (GA) in non-polar organic solvents by using an Ultrastyragel 1000-Å column, isocratically equilibrated with tetrahydrofuran (THF)[1–3]. More recently, an extension of this method has been reported which allows the analysis of the GA conformational equilibrium in more polar organic solvents, (*e.g.*, ethanol[4], and therefore the study of the interaction of this peptide with different metal cations, in particular Ca^{2+}, in terms of the dimer–monomer transition[5]. The advantages of this chromatographic methodology have been discussed previously[4].

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Based on these preliminary results and taking into account the current great interest in the study of hydrophobic self-associating peptides because of their ability to form transmembrane channels in lipid bilayers[6–8], we propose in this paper a novel strategy for the HPSEC analysis of GA, incorporated into artificial phospholipid vesicles (liposomes). The method involves the direct injection of a few microlitres of the aqueous GA-containing liposome suspension into the column, equilibrated with THF, so that the vesicles are immediately disrupted, releasing the polypeptide conformational species to the eluent stream. Evidence is presented that the elution profiles obtained can provide valuable information on some features of the conformational state of GA in the liposome before injection.

On the other hand, since it has been reported that spectroscopic techniques indicate that different conformations of the peptide can exist in the phospholipid bilayers, depending on the method of liposome preparation[9–11], our attention has been primarily focused on relating the information obtained from the chromatographic analysis of the liposomes to a number of variables and experimental parameters involved in the preparation steps: the nature of the organic solvent(s), the incubation time in organic solvent, the class of phospholipid, the "age" of the liposomes, etc. Since one of the most widely used methods of incorporation of GA into the artificial vesicles is the solubilization of both lipid and peptide in an organic solvent (or solvent mixture)[12–15], a study has been first carried out in an organic solvent. The results thus obtained have later been compared with those obtained from liposomes. In addition, a slow GA conformational transition in the liposome has been directly followed as a function of the incubation time of the vesicles, which has shown to be strongly dependent on the type of phospholipid used.

Thus, the proposed HPSEC approach emerges as a rapid and accurate method which helps to provide —especially in combination with other, more conventional spectroscopic techniques— a deeper insight of the properties and organization of GA and other natural or synthetic self-associating polypeptides, incorporated into model membranes.

EXPERIMENTAL

Apparatus

The liquid chromatograph consisted of an M510 solvent-delivery system, an U6K universal injector and a 490 programmable multiwavelength detector, all from Waters Assoc. (Milford, MA, U.S.A.). The system was equipped with a 100-nm pore-size Ultrastyragel column (Ultrastyragel 1000 Å, 30 cm × 0.78 cm I.D.) from Waters. The elution profiles were recorded on a dual-channel recorder (Yokogawa Electric Works, Tokyo, Japan).

Fluorescence experiments were performed in a MPF-44B spectrofluorometer (Perkin-Elmer, Überlingen, F.R.G.) with automatic correction of excitation or emission spectra. The excitation was at 297 nm, and the emission intensity was recorded at 330 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, Königshofen, F.R.G.).

Chemicals and reagents

Gramicidin A (natural mixture) was supplied by Koch Light Labs. (Buckinghamshire, U.K.) and was used as received. Egg yolk phosphatidylcholine (PC) was from Merck (Darmstadt, F.R.G.) and purified according to the column chromatographic method of Singleton *et al.*[16]. The phospholipid gave a single spot when analysed by thin-layer chromatography. L-α-Phospatidyl-L-serine (PS) from bovine brain, tripalmitin, triolein, palmitic acid and cholesterol were obtained from Sigma (St. Louis, MO, U.S.A.) and were used without further purification. All solvents (THF, chloroform, ethanol, methanol) were of spectroscopic grade (Merck). They were passed through a 0.45-μm regenerated cellulose filter (Filtration System, Dublin, CA, U.S.A.) before use.

Procedures

All chromatographic experiments were conducted at room temperature. The column was always eluted isocratically with THF at a flow-rate of 1.0 ml/min. The injection volume was 2 μl, unless otherwise stated.

GA samples were prepared in THF, as described previously[1,2], and stirred for 1 min immediately after preparation, both in the absence and presence of lipid. 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. GA samples, prepared in ethanol, methanol or chloroform–methanol (2:1, v/v), were stirred for 30 s, and aliquots were taken at different times and injected. Aliquots from the same samples were used when combinations of chromatographic and fluorimetric measurements were made.

Dispersions of small unilamellar vesicles (SUVs) were prepared as follows; both PC (or PS) and GA were dissolved in an organic solvent by mixing identical volumes (each 100 μl) of stock solutions. After a given time of incubation of the orgnanic GA phospholipid solution, which varied from a few seconds (referred to as zero time) to 15 h, the solvent was rapidly evaporated under a nitrogen stream and then under high vacuum overnight to remove any traces of solvents. The samples were then hydrated by the addition of 1 ml of Millipore ultrapure water, incubated for 10 min (unless otherwise stated) on ice. The sonicated material was then centrifuged for 15 min at 35 000 *g* to remove probe particles and the remaining multilamellar liposomes. The lipid/polypeptide mole ratio was 50:1. Other details of the experimental conditions used can be found in the corresponding Figure legends.

RESULTS AND DISCUSSION

Characterization of gramicidin A in organic solvents

Since the current methods of preparation of GA-containing liposomes make indiscriminate use of a relatively wide variety of organic solvents (or mixture), varying their polarity and chemical nature, we first studied the peptide conformational equilibrium as a function of incubation time in a set of solvents typically reported in the literature[9,11–15]. Fig. 1 shows as an example the elution profiles of GA samples at a concentration of 0.074 mg/ml, in THF, ethanol, chloroform–methanol (2:1, v/v) and methanol, once equilibrium had been reached.

As previously described[1–4], the peak eluted at 7.9 ml corresponds to intertwined

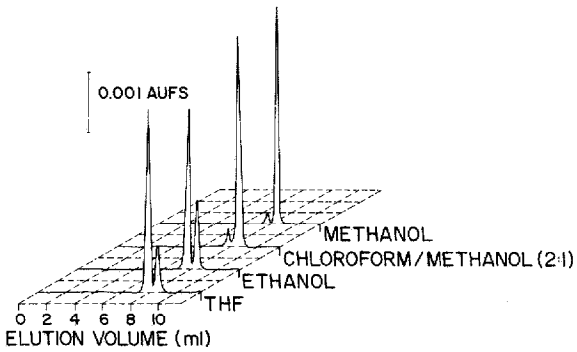

Fig. 1. Elution profiles of GA samples in different organic solvents. The samples (0.074 mg/ml) were prepared by directly dissolving the peptide in the organic solvent. After equilibrium had been reached, 2 μl were injected in all cases. Column: Ultrastyragel 1000 Å. Mobile phase; THF; flow-rate, 1.0 ml/min.

double-helical dimers, whereas that at 8.4 ml corresponds to monomers. As expected, the ratio of both conformational species dramatically varies with the solvent. In the less polar THF the predominant species at equilibrium is the dimer, whilst the monomer predominates in methanol. Fig. 2 shows the kinetic results for GA monomerization (expressed as the disappearance of double-helical dimers) in the aforementioned solvents, at 0.74 mg/ml peptide concentration. This GA concentration is the same as that used later in this work in organic solvents during the preparation of liposomes. Note that at zero time there is 100% of dimeric species because this is the conformation of the commercially available solid gramidicin sample[1]. The mass fraction of each species was directly evaluated from the heights of the peaks, as reported elsewhere[1,2]. Both elapsed time and an increase in solvent polarity result in a shift towards monomeric forms (a decrease in the mass fraction of dimers). It is clear that the time needed for equilibrium to be reached can vary from more than 20 days in THF to a few minutes in methanol. This is of particular interest, as will be later shown, since numerous methods of preparation of liposomes currently reported in the

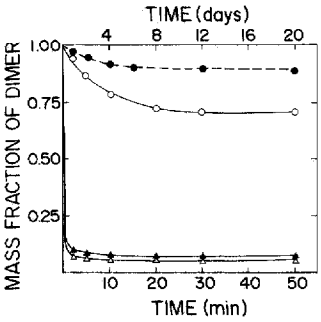

Fig. 2. Kinetic profile of the disappearance of GA double-helical dimers in THF (●), ethanol (○), chloroform–methanol (2:1, v/v) (▲) and methanol (△). The GA concentration was 0.74 mg/ml in all cases. The mass fractions of dimers, calculated from the peak heights in the chromatograms, are plotted *vs.* the time at which each aliquot was taken. The solid lines correspond to the lower axis, and the dotted line to the upper one.

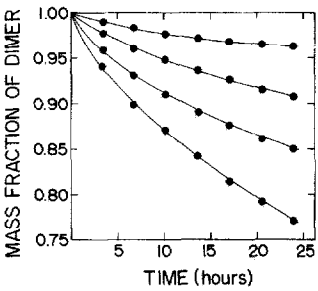

Fig. 3. PC-induced time-dependent monomerization of GA in THF. GA concentration was always 0.74 mg/ml. PC concentrations from top to bottom were: 0, 0.20, 0.33 and 0.50 mg/ml.

literature describe the incubation of the peptide in a given organic solvent during a period of time that is usually not well defined or rather arbitrarily chosen. However, even at short incubation times, the ratio of conformational species strongly depends on the solvent used (see Fig. 2). On the other hand, it was verified by means of a previously reported procedure[2] that the chromatographic results in Fig. 2 in all cases fitted a simple equilibrium model, dimer $\rightleftharpoons$ 2 monomers (results not shown).

It is well known that one of the classical methods of preparation of GA-containing liposomes consists of the simultaneous solubilization of a lipid and a peptide in an organic solvent (or mixture of solvents), followed by complete removal of the solvent and further hydration of the remaining mixed film[12–15]. Since different classes of phospholipids as well as mixtures of phospolipids with other types of lipids have been widely used for solubilization, and based on our previous observation that PC induces a time-dependent monomerization of GA in non-polar solvents[1,2], the influence of different lipid classes on the peptide conformational equilibrium in THF was next investigated. Fig. 3 shows the results obtained for the mass fraction of dimers as a function of time at 0.74 mg/ml GA for different PC concentrations. Similar to the behaviour at other GA concentrations[2], an increase in the phospholipid concentration results in a higher extent of dimer dissociation for the same incubation time. However, the equilibrium proved to be sensitive to the class of lipid used, as demonstrated by the results summarized in Table I, where a series of lipids differing in the type of polar head, chain length and degree of unsaturation are compared by means of HPSEC and fluorescence emission spectroscopy (taking advantage of the Trp residues of GA). All measurements were carried out after incubation for 10 days, the peptide concentration being as low as 0.025 mg/ml to allow reliable spectroscopic data. As deduced from the chromatographic results, the GA conformational equilibrium is drastically altered by PC towards monomeric forms, whereas it is not significantly affected by any other of the lipids assayed. This is supported by the fluorescence measurements where a clear enhancement of the peptide emission intensity (which was previously related to a strong lipid polar head–peptide interaction[2]) is again observed only for PC. Note that in the case of PS, altough the ratio of conformational species is not significantly altered, some fluorescence quenching occurs as the phospholipid concentration increases (Table I). A possible explanation for this will be given below.

However, the PS–GA system in THF proved to be particularly interesting, from the chromatographic point of view, as revealed by the elution profiles obtained. Fig.

4A depicts some actual chromatograms of PS–GA mixtures as a function of the phospholipid concentration. On the one hand the ratio of the heights of dimer and monomer peaks remains invariable (and so does the mass fraction of each species), on the other hand a peak emerging at 6.3 ml has an area which increases linearly with PS concentration, as evidenced in Fig. 4B. The expected molecular weight corresponding to a species at such an elution volume, when interpolated in the column calibration, is higher than 10^4. This suggests the presence of PS reversed micelles, presumably stabilized in THF by a strong interaction among the phospholipid polar-head dissociable groups (the PS contains an additional carboxylic group in its polar moiety relative to the PC). This assumption is supported by: (i) our own experimental chromatographic observations using other charged phospholipids (such as phosphatidic acid, results not shown), which also give in THF a peak at *ca.* 6.5 ml, and (ii) literature data indicating that natural phospholipids can exist in certain non-aqueous solvents as reversed micelles[17,18]. The PS reversed micelles seem to exclude any peptide molecule from their polar interior (since the absolute heights of the GA peaks do not change either) so that the conformational equilibrium is practically unaltered. On the other hand, these observations seem particularly exciting, because, as far as we know, this is the first time that reversed micelles of natural amphiphiles such as phospholipids are eluted from a high-performance gel permeation support, namely Ultrastyragel 1000 Å, and it undoubtedly offers new possibilities for further studies with reversed micelles of both natural and synthetic surfactants. It must be pointed out that the data in Fig. 4B do not allow a reliable determination of the critical micelle concentration (c.m.c.) for PS in THF, though the value appears to be relatively low.

Finally, the cause of the GA fluorescence quenching in the presence of PS (Table I) is not completely understood, but taking into account that there is no direct interaction between the lipid polar heads and the peptide, it seems reasonable to suggest that it may be due to a deactivation of the GA fluorophores in the excited state through direct collisions with the micelles. These differences should be taken into account in the preparation of liposomes and, in fact, they will be considered again later in this work.

Characterization of gramicidin A in liposomes

It can be concluded from the results in the previous section that the GA dimer–monomer conformational equilibrium in organic solvents is very sensitive to

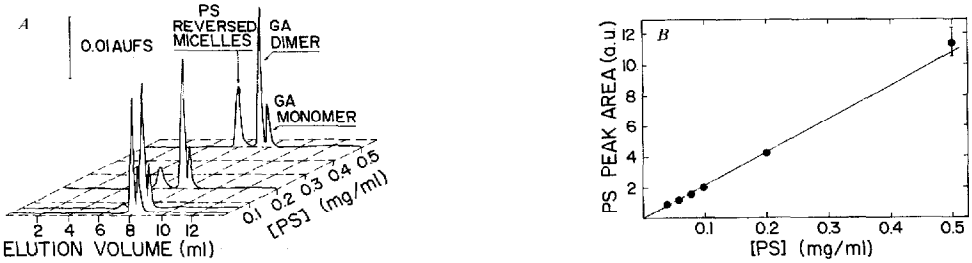

Fig. 4. (A) Elution profiles of GA in THF after 10 days of incubation, as a function of PS concentration. The GA concentration was always 0.025 mg/ml. Injection volume: 75 μl. (B) Plot of the area of the PS reverse micelles peaks (in arbitrary units) *vs.* PS concentration in THF.

a series of factors, such as the nature of the solvent (Figs. 1 and 2), peptide concentration[2], incubation time (Fig. 2), presence of lipid (Figs. 3 and 4A), lipid-to-peptide mole ratio (Fig. 3), lipid class (Table I), etc. Thus, since most of these variables have been used in a rather arbitrary way in the preparation of GA-containing liposomes reported in the literature, and they do affect the actual ratio of conformational species in organic solvents, it seems reasonable to assume that they will also influence the conformational state of the incorporated peptide. In other words, the conformation of the GA in a liposome must be somehow dependent on the "history" of how it was prepared. Altough there is some evidence of this dependence on some of the factors mentioned above[9,11], the characterization of GA incorporated in artificial phospholipid vesicles has been carried out so far only by means of spectroscopic techniques, especially circular dichroism (CD)[9,11,14,19–20]. The latter has shown that the CD spectra are not always identical but vary largely with the incorporation method and the particular experimental conditions used[9–11,14]. For example, it has recently been reported that the solvent-determined conformation of gramicidin affects some peptide properties in model membranes[11]. In this context, the HPSEC method we propose below can be considered as a new, more direct approach (valuable in itself but at the same time complementary to other spectroscopic techniques) for the monitoring of GA incorporated into liposomes, particularly its relationship with the experimental variables involved in the preparation steps. The chromatographic procedure is based on the use of the same Ultrastyragel 1000-Å column as in the previous section, equilibrated with THF. In this case, a few microlitres of an aqueous suspension of GA-containing liposomes is directly injected so that the phospholipid vesicles will be immediately disrupted on top of the column by the organic solvent, releasing both the peptide conformational species and the lipid molecules to the eluent stream. As previously reported[21], the PC molecules strongly interact with the polystyrene–divinylbenzene matrix of the support, thus being dramatically retarded during elution. However, with respect to GA, it must be pointed out that by itself this chromatographic technique does not allow a distinction between monomers and head-to-head dimers (two juxtaposed monomers)[10], since these latter species are dissociated to monomers during elution with THF[1,2]. Thus, it must be inferred that the peak referred to as monomers in the chromatograms obtained from experiments with liposomes may contain a mixture of GA molecules originally present in the liposome as both actual monomers and head-to-head dimers. Nevertheless, since the transition from double-helical dimers (peak eluted at 7.9 ml, see Fig. 1) to monomers in THF is extremely slow, as compared to the *ca.* 10 min of elution, the percentage of double-helical dimers in the chromatogram can indeed be considered as a very reliable, accurate measure of the actual percentage of these species in the original phosholipid vesicles before injection. This parameter was, in fact, used as a probe to test the influence of the experimental conditions during liposome preparation.

The first project of interest was to use the proposed strategy to find out how different organic solvents used for the simultaneous solubilization of lipid and peptide affect the organization of GA incorporated in the liposome. Fig. 5 depicts the elution profiles corresponding to the injection of 2 μl of aqueous suspension of liposomes, prepared from PC + GA in the same solvents as those used in Fig. 1. The vesicles were injected immediately after sonication and centrifugation. As is seen, two symmetrical, perfectly resolved peaks are eluted at 7.9 and 8.4 ml, their elution volumes coinciding

exactly with those of the double-helical dimer and monomer respectively, observed after injection of solutions of GA in organic solvents (see Fig. 1).

This validates the above assumption about the mechanism of disruption of the vesicles on top of the column and the elution of the GA species. Note that if the liposome had not been clearly disrupted immediately after injection, or if some interaction between GA and PC had occurred during elution, both events should have resulted in a shift of the elution volumes, a poor resolution and/or a distortion of the peaks. In addition, it was verified that essentially the same elution profiles were obtained, by direct injection of 2 μl of aqueous liposome suspension, as were obtained when liposomes were diluted 1:25 (v/v) in THF, stirred for 10 s and 50 μl of the resulting solution were immediately injected. The water in the injected sample appeared at 11.5 ml (not shown), as monitored with a refractive index detector.

Interestingly, the observed dimer/monomer ratio drastically depends on the organic solvent using during the preparation of the liposome (Fig. 5). The double-helical dimer (probe) again predominates in the liposomes prepared by simultaneous solubilization of PC and GA in the less polar solvent (THF), whereas it is clearly a minor species in those obtained from the much more polar methanol. In the vesicles prepared from ethanol the situation is intermediate, but still the dimers are predominant. Thus, there seems to exist a clear correlation between the organization of the peptide conformational species in the liposomes, as determined by HPSEC, and the original position of the GA dimer–monomer equilibrium in the organic solvent before evaporation (Fig. 1), so that the more polar the starting organic solvent(s), the lower is the proportion of double-dimeric forms in the vesicles. The fact that the mass fraction of dimers in the liposomes prepared from THF and ethanol (Fig. 5) is slightly lower than that in the corresponding original organic solvents (Fig. 1) can be explained by taking into account that during the solubilization, even if the incubation time is very short, some lipid-induced time-dependent GA monomerization may occur (see Fig 3), especially in this case, where the lipid-to-peptide mole ratio is high. This phenomenon will be considered later in more detail. However, lipid-induced monomerization does not appear to take place when methanol is present in the organic medium, probably due to the cancellation of PC–GA interactions because of the "strength" of this solvent.

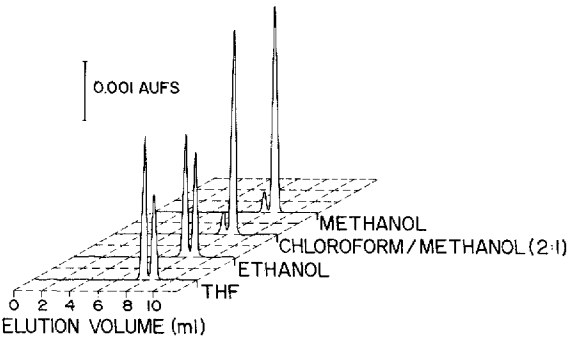

Fig. 5. Elution profiles of fresh samples of GA-containing liposomes, injected immediately after sonication and centrifugation of the vesicles. The liposomes were prepared by solubilizing the lipid and the peptide together in the organic solvent. The final GA concentration in the vesicles was 0.074 mg/ml. The injection volume was 2 μl in all cases. Column: Ultrastyragel 100 Å. Mobile phase: THF; flow-rate, 1.0 ml/min.

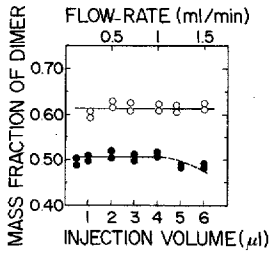

Fig. 6. Influence of the eluent flow-rate (○) and the injection volume (●) on the observed mass fraction of double-helical dimers from two different GA-containing liposome samples. GA concentration in both cases was 0.074 mg/ml.

In order to determine the interval of error due to chromatographic variables for the information obtained by means of the proposed HPSEC strategy, we determined whether changes in the flow-rate or the injection volume altered either the resolution between the dimer and monomer peaks or their ratio for a given liposome sample. Fig. 6 shows the results obtained for two liposome samples in which the dimer ratio was chosen to be different in order not to overcrowd the plot. It can be seen that neither a change in the flow-rate from 0.25 to 1.5 ml/min nor a variation in the injection volume from 0.05 to 4 μl caused any significant alteration of the value of the mass fraction of dimer within a ± 0.02 error. A very slight, artifactual decrease in the mass fraction of dimers seems to occur only for an injection volume of 5 μl or larger. It is probably due to an effect of water-induced monomerization on top of the column, since the "actual" concentration of water surrounding the GA in the injector loop is higher. In fact, we have recently shown that the addition of small proportions of water to solutions of GA in THF induces a time-dependent monomerization[3]. On the other hand, the resolution of the peaks was not affected by changes in either the flow-rate or the injection volume (not shown). In the light of these results (see Fig. 6), a flow-rate of 1.0 ml/min and an injection volume of 2 μl were selected as standard conditions for all further experimens. This allows very reliable measurements (because of the great sensitivity of the UV–VIS detector used) without damage to the column packing, as proven by the fact that the resolution after the analysis of hundreds of samples is similar to that shown in Fig. 5.

Finally, since the phospholipid and GA are usually solubilized together during the preparation of liposomes and it has been demonstrated that the peptide dimer–monomer equilibrium in organic solvent may be drastically affected by PC (see Fig. 3 and refs. 1 and 2), the influence of incubation time of the PC + GA mixture in THF on the resultant mass fraction of dimers in the vesicles was next investigated. For this purpose, aliquots were taken at different times from the lipid–peptide solution, the solvent was rapidly evaporated under a stream of nitrogen and then any traces of solvent were completely removed by rotary evaporation under vacuum overnight. The rest of the procedure followed was that described in the Experimental section. In addition, the liposomes, once prepared and sonicated, were injected immediately thereafter (which will be considered zero time for the incubation of the liposomes in water) and after 24, 48 and 72 h. Fig. 7 summarizes the chromatographic results of this experiment (solid lines), expressed in terms of the mass fraction of double-helical dimers as a function of both the incubation time in organic solvent (abcissa axis) and

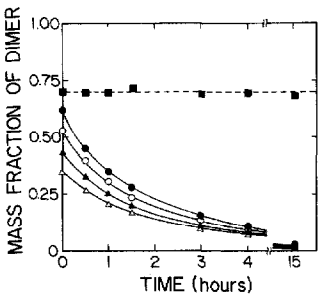

Fig. 7. Solid lines: variation of the mass fraction of double-helical dimers in GA-contaning PC liposomes as a function of both the incubation time of the lipid + peptide mixture in THF (abscissa) and the time of incubation of the vesicle suspensions, 0 (●), 24 (○), 48 (▲) and 72 h (△). The GA concentration was 0.074 mg/ml. Dotted line: mass fraction of dimers in PS liposomes as a function of the incubation time of the lipid + peptide mixture in THF. No significant change in the values was observed after incubation of the vesicles for several days.

the incubation time of the liposomes in water. The longer the incubation in THF, the lower is the rate of disappearance of double-helical dimers in the liposome. This again supports the hypothesis of a correlation between the conformational state of GA in the organic solvent before evaporation (Fig. 3) and the ratio of dimeric/monomeric species observed in the vesicles. In fact, after 15 h of incubation in THF, the remaining dimers account for less than 5% of the total GA present in the liposomes. However, a very interesting observation arises from the fact that the peptide, once the liposome is prepared, undergoes a time-dependent conformational transition, resulting in a slow, progressive decrease in the proportion of double-helical dimers (Fig. 7). This phenomenon, which we have also found to be very sensitive to incubation temperature, and its possible causes are currently under investigation, and our results will be reported elsewhere.

Based on the results in Fig. 4A and in Table I, which show that there is no interaction between PS (as opposed to PC) and GA in THF, and consequently no alteration of the dimer–monomer equilibrium due to the presence of this phospholipid in the organic solvent, it can reasonably be assumed that no variation in the mass fraction of dimers can be observed in PS liposomes, prepared by varying the incubation times of the solubilized PS + GA mixture in THF. The chromatographic results obtained with GA-containing PS vesicles are included in Fig. 7 (dotted line). In this case the liposomes were always injected immediately after sonication and centrifugation. The constant value obtained for the mass fraction of dimers as a function of the incubation time in THF corroborates the above assumption and again confirms the correlation observed in the previous experiments.

It must be pointed out that the large proportion of the mass fraction of "observed" monomers in the chromatograms may actually correspond to Urry's model of head-to-head juxtaposed dimers[22], which would emerge as monomers due to a very weak stabilization by only six hydrogen bonds, as opposed to 30 in the double-helical dimer[2,23]. Although the chromatographic strategy used cannot by itself ascertain the identity of the monomer peak in the starting liposome, we have obtained evidence supporting this assumption from the combined use of HPSEC and CD (not shown). The CD spectra of GA, incorporated into PC liposomes, clearly approach the

spectrum reported by Urry *et al.*[24] as that corresponding to the channel conformation (head-to-head dimer) both when the time of incubation in THF is increased and when the vesicles are incubated at a relatively high temperature (60°C). In both cases a simultaneous HPSEC analysis showed that the mass fraction of observed monomers approaches unity.

It should be emphasized that the results presented so far are preliminary, because they have been obtained for a particular self-associating peptide, GA, with a particular method of preparation of the liposomes. Other experimental factors involved in the preparation of the vesicles clearly remain to be analysed, such as the influence of the lipid-to-peptide mole ratio, the time of sonication of the liposomes, the incubation temperature, the presence of cations in the suspension, etc. Even more interesting is the possibility of comparing the solubilization method used with others also currently reported in the literature, such as the incorporation of GA into pre-formed vesicles, reversed-phase evaporation, etc. A great deal of additional information can be obtained by the combined use of this chromatographic method with other conventional spectroscopic techniques. For example, these approaches may help clarify the cause of the changes observed in the spectra which may be a result of conformational equilibrium when different solvents and different methods of incorporation of the peptide are used[9–11]. On the other hand, this chromatographic technique may prove to be very valuable for investigation a wide variety of other synthetic as well as natural self-associating hydrophobic peptides, incorporated in liposomes. Results of investigations of all these aspects will be the subject of future reports.

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