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Effects of random copolymers of lysine on the thermotropic behaviour of dipalmitoylphosphatidylglycerol vesicles

A fluorescence anisotropy study

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The effects of some random copolymers of lysine on the thermotropic behaviour of phospholipid vesicles, mainly dipalmitoylphosphatidylglycerol (DPPG), were studied by monitoring the steady-state fluorescence anisotropy with 1,6-diphenyl-1,3,5-hexatriene (DPH) as a membrane probe. A characteristic effect of the polylysine-tyrosine 4:1 copolymer was observed: DPPG vesicles are strongly stabilized, the gel-to-fluid transition going from 40°C in the absence to 52°C with an excess of copolymer (corresponding to a lipid/amino-acid ratio $R = 0.5$). For $R > 0.5$, the gel-to-fluid transition shows a three-step profile. This triphasic transition, which appears with a much better resolution than with polylysine, demonstrates the formation of stable distinct domains of reasonable size, coexisting from 41 to 51°C. Such stability, over a temperature range of 10°C, is quite unusual and unexpected for a phase separation of mixed lipid phases. The peculiarity of this copolymer was proved by investigating the interactions of DPPG vesicles with polylysine-tryptophan 4:1, polyornithine-lysine 4:1 and polylysine-tyrosine 1:1. We hypothesize that the observed effects are correlated with the ability of these copolymers to adopt/not adopt an α-helical or β-sheet conformation upon interacting with anionic vesicles.

1. Introduction

Biological membranes are composed primarily of lipids and proteins, and it is therefore important to study their interactions for a better understanding of the factors determining membrane structure and dynamics.

Depending on their nature, proteins are either embedded in the hydrophobic core of the bilayer, in which case they are referred to as intrinsic or integral proteins, or simply bind at the outside of the bilayer, largely by electrostatic interactions, in which case they are referred to as extrinsic or peripheral proteins.

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Most studies of the molecular interactions between lipids and proteins have been performed on model systems composed of well defined lipid and protein components. Poly(L-lysine) is one of the most interesting models of extrinsic proteins because of its basic character. Each lysine group bears a positive charge at physiological pH and so can bind to acidic lipid bilayers.

Carrier and Pezolet [1,2] performed a Raman spectroscopic study of the interaction of poly(L-lysine) with dipalmitoylphosphatidylglycerol (DPPG) bilayers. They found that the nature of the interaction depended on the molar ratio of the constituents. At up to one lysine per lipid molecule, the bilayer was stabilized by the polypeptide which underwent a conformational transition towards an α-helical structure in which the electrostatic interactions were probably maximal.

Carrier et al. [3] used fluorescence polarization to investigate the thermotropic behaviour of DPPG vesicles interacting with polylysines. The molecular weight of the polypeptide appeared to be a key parameter. Long polylysine induced a lateral phase separation for lipid to lysyl ratios (R) greater than unity and a shift of $+4°$ C in the single gel-to-fluid transition was observed when there was excess lysine. With short polylysine ($M_w \leq 4000$), no domain formation was observed and the transition temperature shift was much smaller. According to Carrier and Pezolet [2], those differences between short and long polylysines correlated with the conformation adopted by the polypeptide upon binding to the vesicle, the formation of α-helices being favoured in the case of long polylysines ($M \geq 14000$). X-ray data obtained by the same authors suggested that the basic polypeptide acts as a bridge between neighbouring bilayers, thus causing their aggregation and dehydration.

Incidentally, the ability of polylysine to induce aggregation and, under some conditions, fusion, was demonstrated for unilamellar vesicles composed of anionic phospholipids [4–7]. For fusion to occur after aggregation, optimal conditions consist of charge neutralization without the vesicles being covered with polypeptide. This is consistent with the notion that the polypeptide is necessary for fusion, but limits it by steric hindrance.

These studies have well clarified the mimicry by polylysine of the interaction of an extrinsic protein with phospholipid vesicles. To enable further progress in this domain, it would be useful to have slightly more elaborate models than single homopolymers. One possibility is to use copolymers of lysine and other amino acids. For the time being, only limited investigations have been performed by Bach et al. [8,9], using circular dichroism or differential calorimetry, on copolymers of lysine with tyrosine and phenylalanine. To collect more data on such systems, we have used 1,6-diphenyl-1,3,5-hexatriene (DPH) fluorescence anisotropy to monitor the thermotropic behaviour of lipid bilayers. We investigated the effect of some lysine copolymers on the thermotropic behaviour of unilamellar or multilamellar vesicles containing DPPG, as a function of the lipid/amino-acid residue molar ratio.

2. Methods

2.1. Materials

Phospholipids: dipalmitoyl-L-α-phosphatidyl-DL-glycerol, ammonium salt (DPPG); dipalmitoyl-DL-α-phosphatidyl-L-serine (DPPS); dipalmitoyl-L-α-phosphatidylethanolamine, (DPPE); dipalmitoyl-L-α-phosphatidylcholine, (DPPC) and dimyristoyl-L-α-phosphatidylcholine (DMPC) were obtained from Sigma and used without further purification.

Polylysine and L-lysine random copolymers were obtained from Sigma. Their molecular weights are: polylysine, $M_w = 22000$; poly(Lys, Tyr) 4:1, $M_w = 24000$; poly(Lys,Trp) 4:1, $M_w = 38000$; poly(Orn,Tyr) 4 : 1, $M_w = 23000$; poly(Lys,Tyr) 1 : 1, $M_w = 90000$. Diphenylhexatriene was from Koch-Light Laboratories.

2.2. Fluorescence anisotropy measurements

An SLM-8000 SC spectrofluorometer in the T-format was used to measure the steady-state fluorescence anisotropy r ($r = (I_{\parallel} - I_{\perp})/(I_{\parallel} + 2I_{\perp})$). The excitation wavelength was 350 nm and 435 nm Schott interference filters were used for the emitted light. A home-built device ensured automatic rotation of the excitation polarizer, allowing continuous measurement of r. Cuvette temperature was maintained by a circulating water bath (Haake F3), itself piloted by a temperature programmer (Haake PG 10). The rate of heating was 1° C/min and the temperature was continuously monitored by a thermocouple inserted into the cuvette to a level just above the light beam. Thus, the temperature profiles of fluorescence anisotropy were directly obtained on an XY recorder (Sefram TGM 164), with about three points by degree (not all points are plotted in the figures).

2.3. Vesicle preparation

To obtain multilamellar vesicles, aliquots of stock phospholipid solutions in $CHCl_3$ were added to a 50 ml round-bottom flask and the volume was brought to 5 ml with chloroform. The sample was then dried as a thin film by rotary evaporation,

and the vacuum maintained for 30 min after complete evaporation to remove any residual solvent. The lipid film was dispersed in buffer (100 mM phosphate, 10 mM EDTA, pH 7) at a temperature 10 °C higher than the transition temperature of the phospholipid, by vigorous vortex shaking for 2 min. Small unilamellar vesicles were obtained by sonicating, at the same temperature in a bath-type sonicator (Bransonic 320, 120 W), the multilamellar vesicle dispersion for 90 min. Large unilamellar vesicles were prepared by the reverse-phase evaporation procedure described by Szoka and Papahadjopoulos [10]. As all the phospholipids used have saturated acyl chains, no special precautions against oxidation were taken.

DPH was dissolved in tetrahydrofuran (2×10^{-3} M) and then dispersed in buffer to obtain a final concentration of 2×10^{-6} M. Labelling of vesicles was carried out by mixing an equal volume of DPH dispersion with a suspension of vesicles at

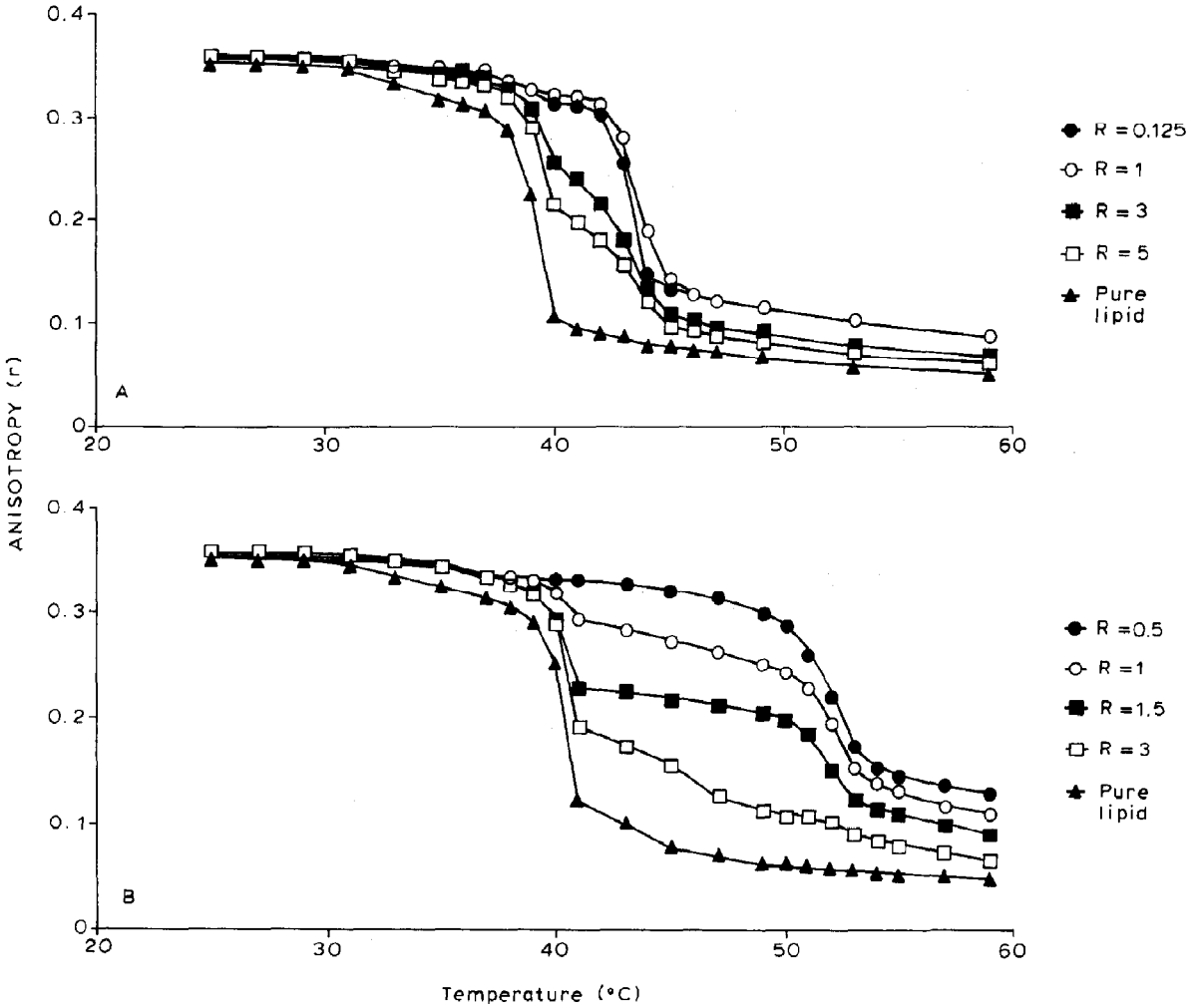

Fig. 1. Temperature profiles of fluorescence anisotropy of DPH embedded in DPPG multilamellar vesicles in the presence of poly(L-lysine) (A) or poly(Lys,Tyr) 4:1 (B) at different lipid/amino-acid ratios (R).

2×10^{-4} M in phospholipids. To allow complete incorporation, the samples were incubated for 30 min prior to complexing with the copolymers.

The complexes of vesicles with copolymers were obtained by diluting 0.3 ml of a vesicle suspension at 10^{-4} M in lipids with $(2.7 - v)$ ml of buffer, and then adding v ml of a stock solution of copolymer at 10^{-3} M in amino acid (v determining the lipid/amino-acid ratio R). This last solution was incubated for 15 min at room temperature before starting a heating scan. All the copolymers used were soluble in the buffer, except poly(Lys,Tyr) 1 : 1 which must be dissolved at pH 11. In this case, because only small volumes of copolymer stock solution were added, the final pH was scarcely affected, remaining at 7.

3. Results

3.1. Results for poly(Lys,Tyr) 4 : 1

3.1.1. Interaction with DPPG vesicles

Fig. 1A shows the temperature profiles of DPH fluorescence anisotropy when polylysine was ad-

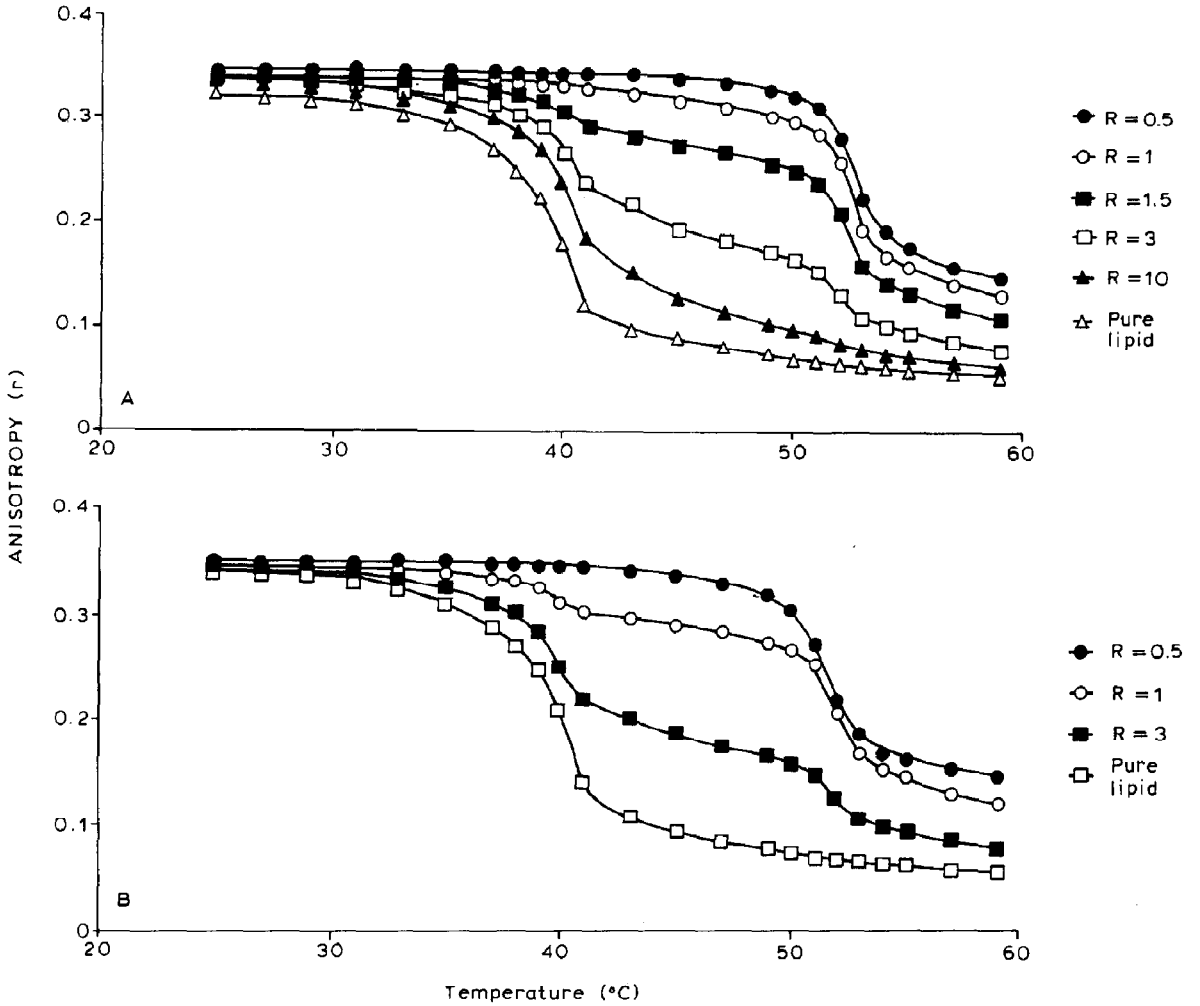

Fig. 2. Temperature profiles of fluorescence anisotropy of DPH embedded in DPPG small unilamellar (A) or large unilamellar (B) vesicles in the presence of poly(Lys,Tyr) 4 : 1 at different R.

ded to DPPG multilamellar vesicles. The curves obtained are identical to those obtained by Carrier et al. [3] with a longer polylysine ($M_w = 100\,000$). The gel-to-fluid transition comprises three steps, indicating the emergence of three distinct phases within the bilayer. With poly(Lys,Tyr) 4:1, as shown in fig. 1B, this triphasic transition appears with a much better resolution than with polylysine: the bilayer is stabilized from 40°C in the absence to 52°C with an excess of polypeptide ($R = 0.5$), so that the intermediate step, which appears ideally flat for $R = 1.5$, is maintained over 10°C. Furthermore, the anisotropy in the fluid phase, after the transition, is higher with an excess of poly(Lys,Tyr) 4:1 ($r = 0.145$ at 55°C) than with polylysine ($r = 0.09$).

As shown in fig. 2, the curves obtained with small or large unilamellar vesicles indicate the same behaviour, except for the lower cooperativity of the first transition, which is well known from comparison of unilamellar and multilamellar vesicles.

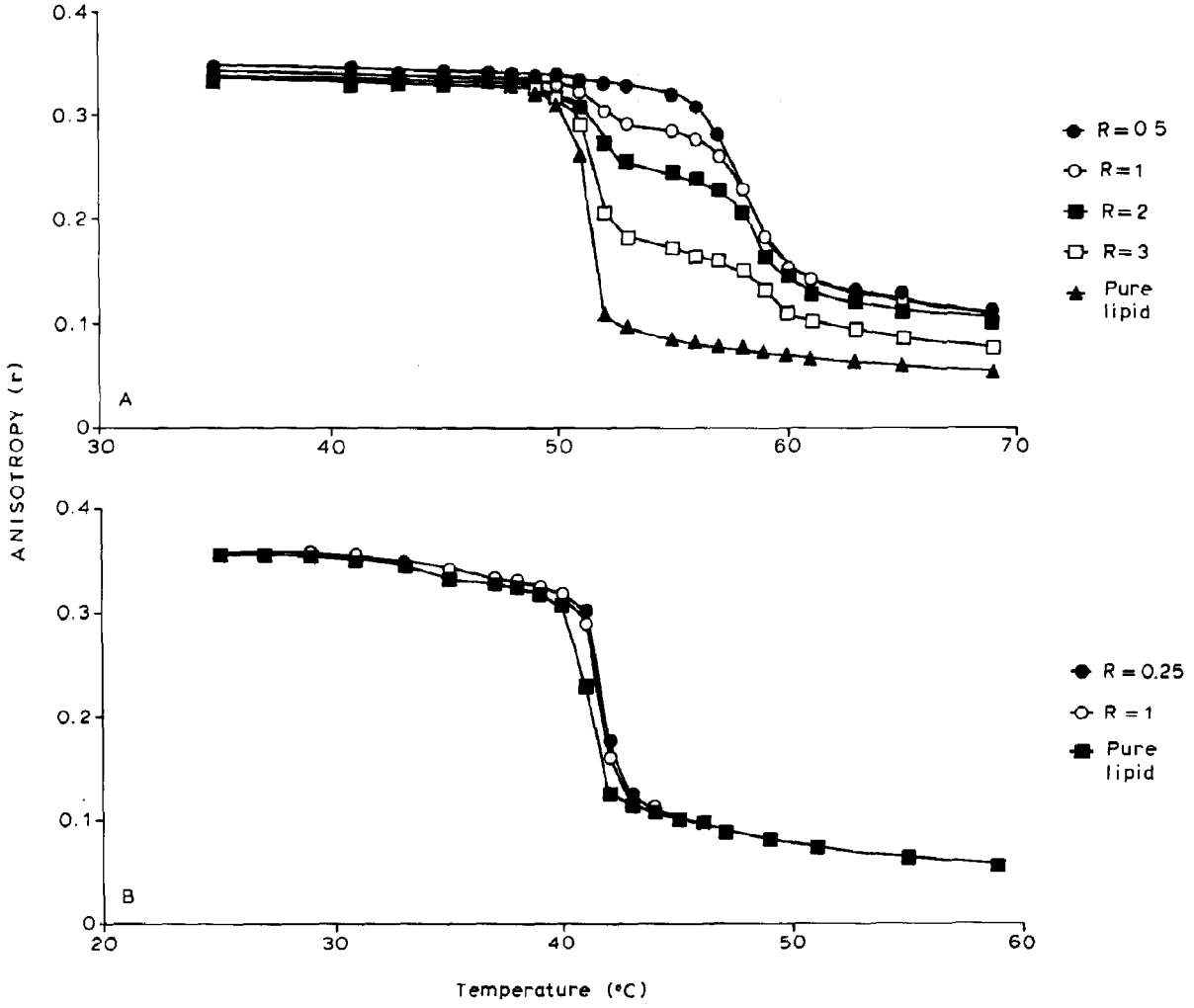

Fig. 3. Temperature profiles of fluorescence anisotropy of DPH embedded in DPPS (A) or DPPC (B) multilamellar vesicles in the presence of poly(Lys,Tyr) 4:1 at different R.

3.1.2. Interaction with DPPS and DPPC vesicles

The temperature profiles for these two types of vesicles are shown in fig. 3. With DPPS multilamellar vesicles, the curves greatly resemble those obtained with DPPG: a well characterized three-step gel-to-fluid transition, and a high ΔT at saturating R. The ΔT observed in this case is only about 8° C, less than the 12° C observed with DPPG vesicles, however, one should bear in mind that the transition temperature of pure DPPS itself (51° C) [10] is 10° C higher than that of pure DPPG itself (40° C) so that the transition curve for $R = 0.5$ is shifted to 58° C for DPPS vesicles as against 52° C for DPPG vesicles. In contrast, with DPPC multilamellar vesicles, poly(Lys,Tyr) 4:1 has little effect ($\Delta T < 2$° C).

3.1.3. Vesicles containing two phospholipids

The influence of poly(Lys,Tyr) 4:1 on two-component (50% vs. 50%, molar) multilamellar vesicles, one of them being DPPG, was investigated. The second component was a non-anionic phospholipid, either DPPE ($T_c = 60$° C) or DMPC ($T_c = 23$° C) [10].

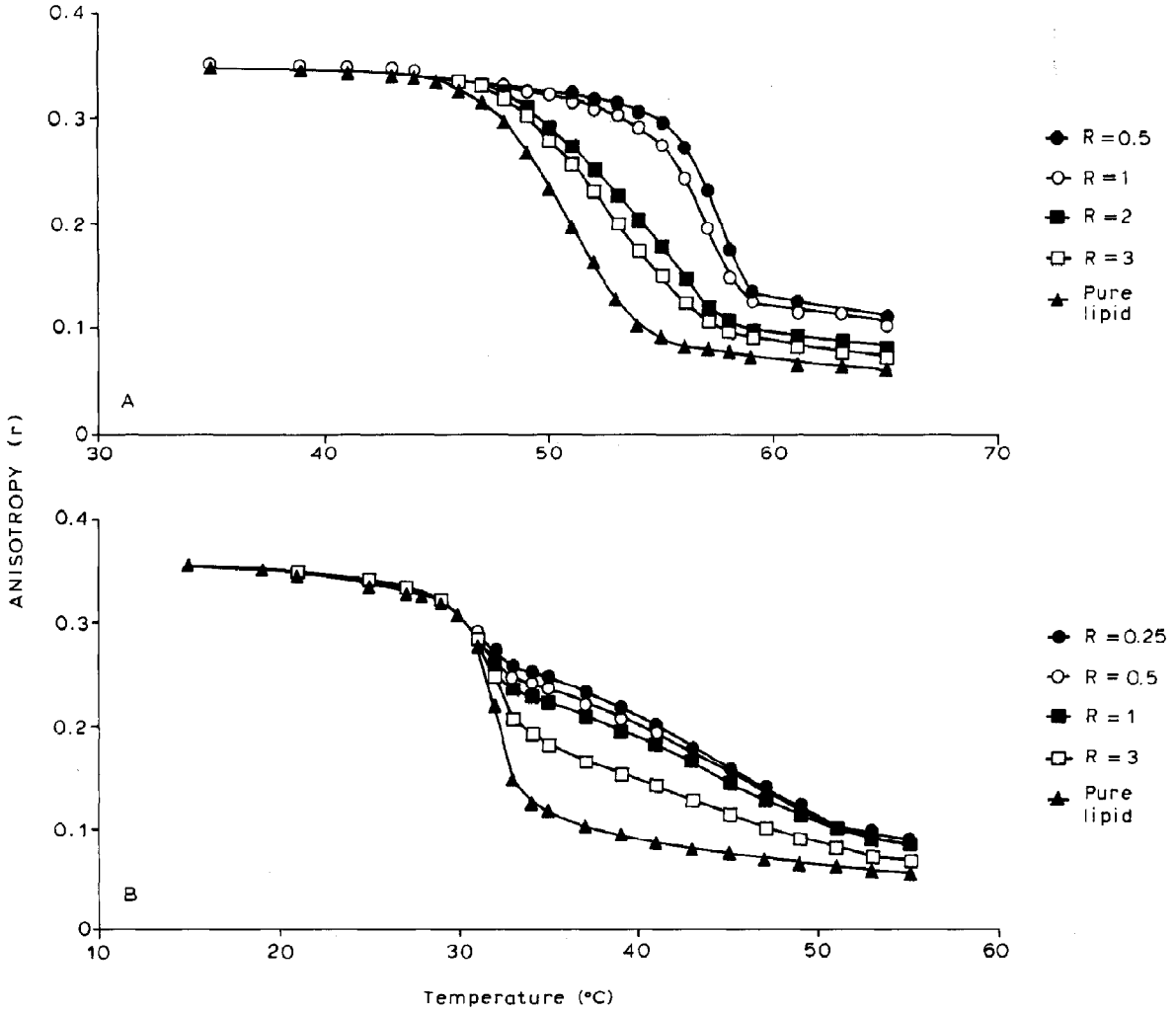

Fig. 4. Temperature profiles of fluorescence anisotropy of DPH embedded in multilamellar vesicles composed of mixtures of DPPE-DPPG (A) or DPPG-DMPC (B), at 1:1 molar ratio, in the presence of poly(Lys,Tyr) 4:1 at different R.

With DPPG-DPPE vesicles (fig. 4A), only one characteristic gel-to-fluid phase transition is seen, irrespective of the value of R, which indicates that the two components are always ideally mixed, the addition of increasing amounts of poly(Lys,Tyr) 4:1 inducing an overall increase in the transition temperature of the vesicles, without any noticeable phase separation.

The behaviour for DPPG-DMPC vesicles is different (fig. 4B): whatever the amount of polypeptide added, the transition temperature of the initial mixture remains present in the fluorescence anisotropy diagram, whereas above this temperature, in the fluid phase, an increase in anisotropy is observed. This anisotropy slowly decreases with increasing temperature, in a non-cooperative way.

3.2. *Other copolymers interacting with DPPG vesicles*

3.2.1. *Poly(Lys,Trp) 4:1*

The temperature profiles of the fluorescence anisotropy for this copolymer interacting with DPPG multilamellar vesicles are shown in fig. 5A.

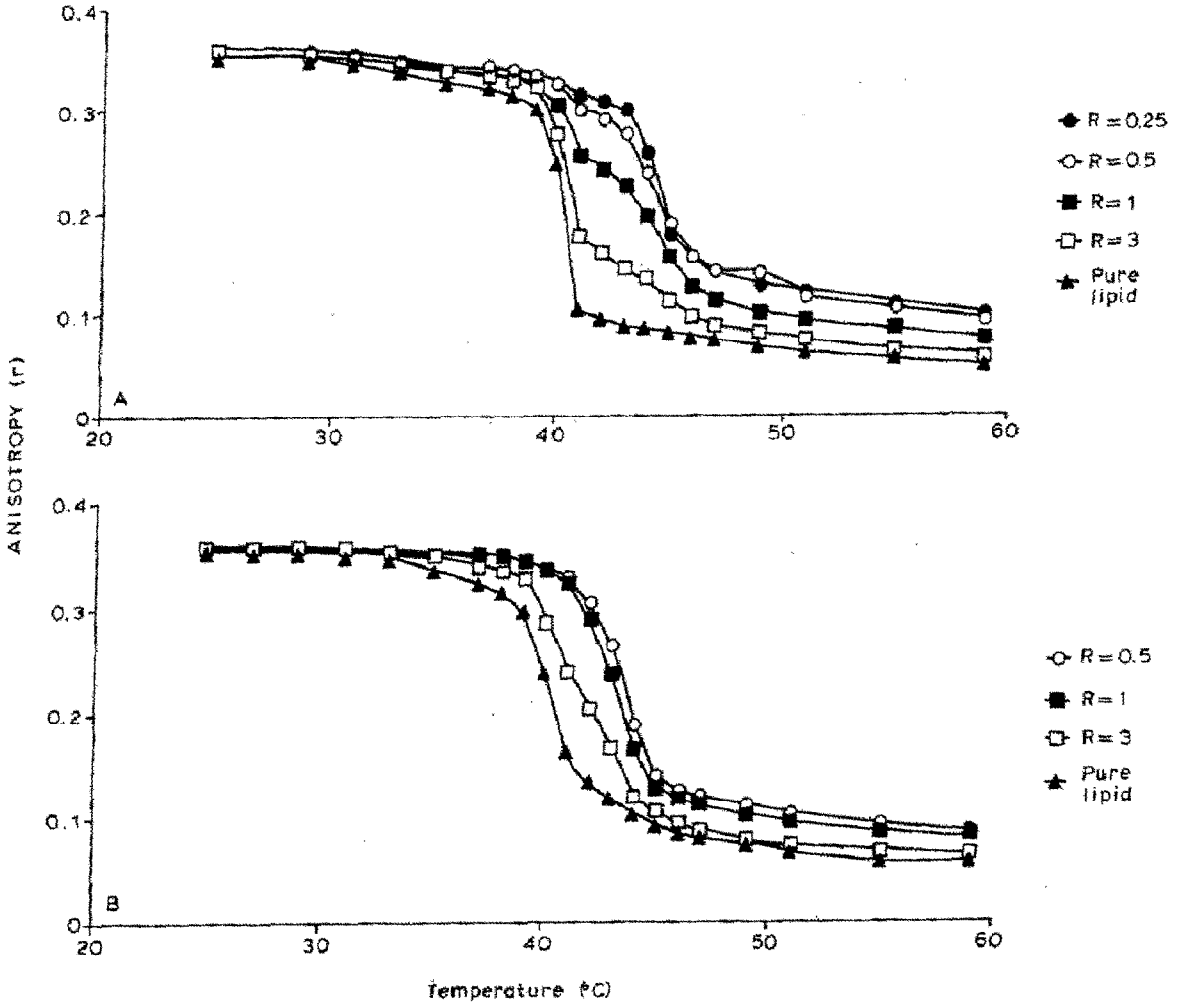

Fig. 5. Temperature profiles of fluorescence anisotropy of DPH embedded in DPPG multilamellar vesicles in the presence of poly(Lys,Trp) 4:1 (A) or poly(Orn,Tyr) 4:1 (B) at different R.

Characteristic three-step transitions, as for poly(Lys,Tyr) 4 : 1, are observed, but the stabilization of the bilayer is only, for $R = 0.25$, $\Delta T = +4°C$ as against $+12°C$ for the foregoing. This effect is of the same magnitude as for a long polylysine.

3.2.2. Poly(Orn,Tyr) 4 : 1

Since ornithine differs from lysine only in having one less methylene group in the side chain of the amino acid, we might expect few differences in the interaction of this copolymer with DPPG vesicles as compared to poly(Lys,Tyr) 4 : 1. This is not so; on the contrary, striking differences appear, as can be seen in fig. 5B: the three-step profile completely disappears with multilamellar vesicles, and the stabilization of the bilayer is much less significant ($\Delta T = +3°C$). Similar results were obtained with small unilamellar vesicles (data not shown).

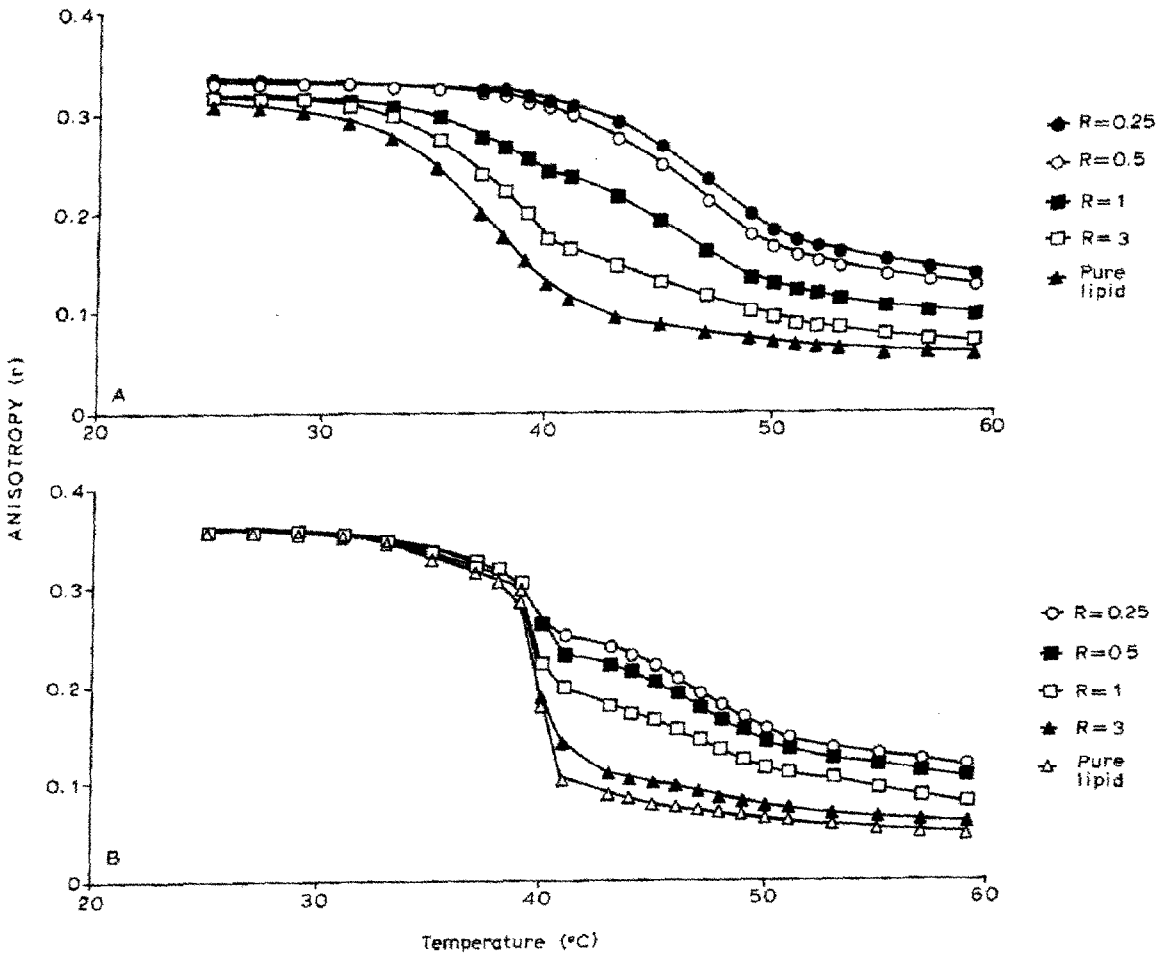

Fig. 6. Temperature profiles of fluorescence anisotropy of DPH embedded in DPPG small unilamellar vesicles (A) or multilamellar vesicles (B) in the presence of poly(Lys,Tyr) 1 : 1 at different R.

3.2.3. Poly(Lys,Tyr) 1 : 1

The curves obtained with this copolymer are shown in fig. 6. The degree of stabilization (ΔT = 10 °C for $R = 0.25$) remains high in the case of small unilamellar vesicles, but the three-step profile has disappeared. With multilamellar vesicles, it could be considered, to some extent, that this triphasic profile persists, but without any cooperativity for the last step, with a coexisting phase whose anisotropy slowly decreases with increasing temperature.

4. Discussion

The results obtained with the copolymer poly(Lys,Tyr) 4 : 1 clearly show significant and characteristic changes in the thermotropic behaviour of anionic vesicles. It has already been shown that polylysines with a high molecular weight [3] induce a three-step gel-to-fluid transition of DPPG bilayers, with good cooperativity for each step, demonstrating the formation of distinct domains of reasonable size. With these long polylysines, the temperature zone at which coexisting phases are visible goes from 40 to 45 °C, these temperatures characterizing pure lipids and totally interacting lipids, respectively. With poly(Lys,Tyr) 4 : 1, the stabilizing effect of this copolypeptide on DPPG vesicles is so strong that the vesicles in complete interaction undergo their phase transition at a temperature 12 °C higher than that of pure DPPG vesicles. Here too, the cooperativity of both phase transitions is particularly good and, for example, for a lipid/amino-acid ratio equal to 1.5, coexisting phases whose average fluidity is practically constant over 10 °C can be observed with these vesicles. Such stability is quite unusual and unexpected for a mixed lipid phase.

The type of vesicles used does not seem to have much influence on the interaction between DPPG and poly(Lys,Tyr) 4 : 1. However, with small unilamellar vesicles, and, to a lesser extent, with large unilamellar vesicles, the addition of copolymer induces an increase in the cooperativity of the first transition, as was previously seen with polylysine [3]. Moreover, at saturation ratio $R = 0.5$, unilamellar and multilamellar vesicles give identical curves, indicating that the unilamellar structure is gradually destroyed as R decreases, leading to a final physical state which is identical for all the samples, probably as a result of aggregation or fusion processes induced by the polypeptide. As mentioned before, the saturation curves are obtained for $R = 0.5$, signifying two amino-acid residues per phospholipid molecule, whereas for polylysine, saturation occurs at $R = 1$. Therefore, if poly(Lys,Tyr) 4 : 1 has a greater stabilizing effect than single polylysine, the stoichiometry of the interaction requires more polypeptide. Here, we probably have a subtle balance between an electrostatic effect due to lysine residues and a hydrophobic effect due to tyrosine. In any case, the interaction between phospholipid vesicles and this copolypeptide is primarily governed by electrostatic interactions, as is obvious from the lack of effect on DPPC vesicles.

The differences in the experiments with the two-component vesicles are easy to explain. With DPPE-DPPG vesicles, we can consider that the phosphatidylethanolamine, like a phosphatidylcholine, is unaffected by the copolypeptide. Taking into account that the transition temperature of pure DPPE-DPPG vesicle is about 50 °C (cf. fig. 4A) and that of pure DPPE, 60 °C [10], the difference in the transition temperatures for the three distinct phases which may exist in this system (mixed DPPE-DPPG, pure DPPE, DPPG interacting with the polypeptide) is always less than 10 °C; this small difference does not allow any phase separation and the mixture in all cases behaves ideally. This is not the case with DMPC-DPPG vesicles, where DMPC melts at 23 °C and the mixture at 32 °C. The difference in transition temperature between unaffected DMPC and DPPG in interaction with the polypeptide is great enough to allow a phase separation, such a mixture being non-ideal. However, perhaps because of the lack of electrostatic interactions, a proportion of DMPC seems to be drawn by the patches of stabilized DPPG, which precludes any constancy in the fluidity (and hence in fluorescence anisotropy) of the corresponding phase [11].

The high stabilizing power coupled with induced phase separation (or segregation), stable over a wide temperature range, appears to be

characteristic of the statistical sequence (4 lysines, 1 tyrosine). As for commercial availability allowed, we tried to change the composition of the sequence either by changing either one component or the lysine/tyrosine ratio.

The first approach offered two possibilities: either replacing tyrosine with tryptophan, or lysine with ornithine. Poly(Lys,Trp) 4 : 1 interacting with DPPG vesicles has the same effects as a long polylysine: clear phase separation, but bilayer stabilization of only +5 °C. The only difference from polylysine is that a saturation effect needs four amino-acid residues for one lipid ($R = 0.25$) with this copolymer, as against equimolarity with polylysine, and $R = 0.5$ with poly(Lys,Tyr) 4 : 1. Thus, replacing tyrosine with tryptophan not only precludes any wide temperature range segregation, but also reduces the effectiveness of the interaction. Ornithine differs from lysine in having one less methylene group on its side chain; the difference this makes to the copolymer is steric and might not affect the electrostatic part of the interaction. The results show that this small steric modification is sufficient to suppress totally the triphasic behaviour of the gel-to-fluid transition and thus the phase separation; the stabilization effect itself falls to +4 °C.

In fact, there is only one possibility for changing the lysine/tyrosine ratio, namely, the use of poly(Lys,Tyr) 1 : 1. This copolymer gives some contrasting results depending on the type of vesicle used. With small unilamellar vesicles, only strong stabilization is observed ($\Delta T = +10$ °C) without any phase separation. With multilamellar vesicles, there is always a fraction of DPPG which remains unaffected, since a transition at 40 °C always persists, even at low R. Increasing the copolymer concentration progressively rigidifies the fluid phase above the transition temperature. The reason for this may be that not all of the bilayers of a multilamellar vesicle are accessible to this copolymer; this would constitute a notable difference from poly(Lys,Tyr) 4 : 1. More experiments, in particular studies of the aggregation and fusion processes induced by these copolypeptides, are needed to confirm this.

The basic fact which emerges from all these fluorescence anisotropy measurements is the strong stabilizing effect of poly(Lys,Tyr) 4 : 1, together with the wide temperature range over which two phases coexist: pure phospholipids and phospholipids interacting with the polypeptide. With polylysines, it has already been reported that the binding involves some hydrophobic effects in addition to the initial electrostatic interaction [1,2] and therefore we must consider structural implications for this copolymer.

According to Raman spectroscopy [1,2] and circular dichroism data [12], long polylysine goes from a random coil to an α-helix structure upon binding to anionic vesicles; this transconformation does not occur with short polylysines. Consequently, it seems that a polypeptide which does not adopt an α-helix structure upon binding will not have similar effects on the thermotropic properties of the phospholipid vesicles. In contrast, it is likely that poly(Lys,Tyr) 4 : 1, which gives rise to stronger stabilization and phase separation, might be even more prone than long polylysine to adopt an α-helical or eventually a β-sheet structure upon interacting with anionic bilayers.

In any case, it is clear that, with all the copolymers used in this study, we are in position to check the hypothesis of a correlation between phase separation appearing in the bilayer and conformational change of the polypeptide from a random coil to an α-helix or β-sheet structure. Circular dichroism studies are planned to investigate further this assumption. Another interesting application from this work would be to determine whether poly(Lys,Tyr) 3 : 1 or 2 : 1 induces similar phase separation and stabilization of anionic bilayers. These copolymers are not yet commercially available, but they could be obtained by organic synthesis. Equally interesting, but rather more difficult, would be the study of regularly sequenced copolymers rather than the statistical samples used in this work.

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References

1 D. Carrier and M. Pezolet, Biophys. J. 46 (1984) 497.
2 D. Carrier and M. Pezolet, Biochemistry 25 (1986) 4167.
3 D. Carrier, J. Dufourcq, J.F. Faucon and M. Pezolet, Biochim. Biophys. Acta 820 (1985) 131.
4 A.E. Gad, B.L. Silver and G.D. Eytan, Biochim. Biophys. Acta 690 (1982) 124.
5 A.E. Gad, Biochim. Biophys. Acta 728 (1983) 377.
6 A.E. Gad, M. Bental, G. Elyashiv and H. Weinberg, Biochemistry 24 (1985) 6277.
7 A. Walter, C.J. Steer and R. Blumenthal, Biochim. Biophys. Acta 861 (1986) 319.
8 D. Bach, K. Rosenheck and I.R. Miller, Eur. J. Biochem. 53 (1975) 265.
9 D. Bach and I.R. Miller, Biochim. Biophys. Acta 433 (1976) 13.
10 F. Szoka, Jr and D. Papahadjopoulos, Annu. Rev. Biophys. Bioeng. 9 (1980) 467.
11 E.J. Findlay and P.G. Barton, Biochemistry 17 (1978) 2400.
12 G. Hammes and S.E. Schullery, Biochemistry 9 (1970) 2555.