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Association of polyene antibiotics with sterol-free lipid membranes: I. Hydrophobic binding of filipin to dimyristoylphosphatidylcholine bilayers

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Abstract

The interaction of filipin III with multilamellar vesicles (MLV) of dimyristoylphosphatidylcholine (DMPC) was studied by four complementary methods leading to the following results: (1) The modifications of the filipin dichroic spectrum, by adding preformed fluid DMPC MLV, provide evidence of a saturable association with the stoichiometry DMPC/filipin = 4.2 ± 0.5, constant between 24 and 35°C. (2) Thermograms obtained by differential scanning calorimetry (DSC) on mixtures where filipin is incorporated during the formation of MLV exhibit a high-temperature tail the more marked the higher the filipin content and some structures at temperatures which depend on this content. The corresponding evolution with the temperature of the CD spectra reveals that the characteristic bound filipin spectrum appears at the temperature at which a structure emerges. (3) Titration calorimetry measurements reveal that the association process is exothermic in the temperature range of the DSC endotherms in agreement with the filipin-induced ordering of the lipid chains, previously established by ^{2}H-NMR in the same temperature range (Milhaud et al. (1989) Eur. Biophys. J. 17, 151–158). A discussion of the relevancy of this exothermicity to the hydrophobic effect is developed by referring to the paper by Wimley and White ((1993) Biochemistry 32, 6307–6312).

Keywords: DSC; Titration calorimetry; Circular dichroism; Multilamellar vesicle; Filipin III; Dimyristoylphosphatidylcholine; Lipid bilayer

1. Introduction

Contrary to polyene antibiotics such as amphotericin B or nystatin, filipin is not used as a drug because of its toxicity towards animal cells. It is rather used as a cholesterol probe insofar as it interacts with cholesterol-containing membranes, by forming protrusions visible by electron microscopy [1–3]. Such protrusions have been connected to a gathering [4] and an immobilization [5] of the membrane cholesterol. On the other hand, in membranes where the filipin-to-phospholipid molar ratio, R, reaches a few per cent it has been established that filipin binds to the phospholipid matrix itself, in competition with its specific binding to membrane cholesterol [6,7]. At these R values, filipin promotes on fluid cholesterol-free DMPC bilayers the formation of domains where the acyl chains have the same order degree as in the gel phase; these domains have been assigned to filipin-DMPC association complexes [8]. Moreover, at such R values, the addition of filipin to cholesterol-free vesicles provokes the permeabilization of the vesicle membranes to alkaline ions [9]; this permeabilization has been attributed to the coexistence of gel and fluid domains owing to the large density fluctuations in the gel/fluid interfaces [9].

We have anticipated from a preliminary study by DSC on the interaction of filipin and nystatin with DPPC, DMPC and DLPC MLV [10] that all the polyene antibi-

Abbreviations: UV, ultraviolet; DSC, differential scanning calorimetry; CD, circular dichroism; NMR, nuclear magnetic resonance; MLV, multilamellar vesicles; SUV, small unilamellar vesicles; DPPC, dipalmitoylphosphatidylcholine; DMPC, dimyristoylphosphatidylcholine; DMPA, dimyristoylphosphatidic acid; DLPC, dilauroylphosphatidylcholine; DMSO, dimethylsulfoxide; T_m, temperature of the main transition of phospholipids.

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otics can promote an ordering of the lipid chains of the membrane phospholipids and that it would be all the more marked as the association with the phospholipid is tight. Consequently, we have undertaken a comprehensive study of the interaction of polyene antibiotics with different saturated phosphatidylcholines in order to determine if structural molecular parameters such as the acyl chain length play a role in the tightness of the association.

In the present study, four complementary methods were used: DSC, focused on the state of the membrane, CD and UV absorption, on that of the antibiotic and titration calorimetry on the thermodynamics of the antibiotic–membrane interaction.

2. Materials and methods

2.1. Materials

DMPC and filipin III were purchased from Sigma (St. Louis, MO) and used without further purification. When filipin was added as organic solutions its concentration was deduced from UV absorption ($\varepsilon_{345} = 9.2 \cdot 10^4$ M^{-1} cm^{-1}, in DMSO and $\varepsilon_{337.5} = 8.9 \cdot 10^4$ M^{-1} cm^{-1} in methanol). For aqueous solutions, a 10 mM Hepes (pH 7.2) buffer was used.

2.2. Lipid bilayers

The most thermodynamically stable form of lipid vesicles, namely MLV, was used. The desired amount of phospholipid, as a chloroformic solution, was evaporated under a nitrogen flow. The dried film was desiccated for several hours in vacuum and dispersed in the buffer, with a bath sonifier, for around 10 min, at 10°C above T_m. The lipid phosphorus content of the vesicles was determined according to Stewart [11].

For titration calorimetry experiments, 5 mol% of DMPA was added to DMPC before the MLV preparation, in order to prevent any flocculation of the lipid suspension in the

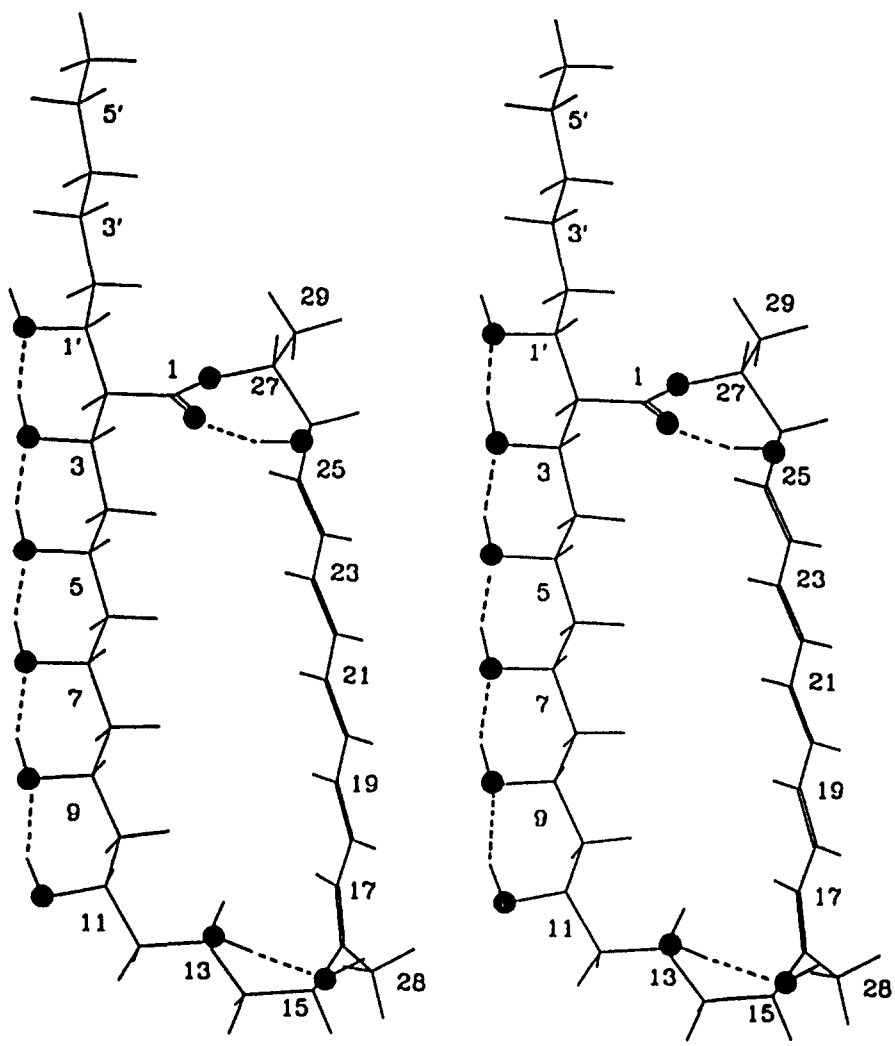

Fig. 1. Stereoview of a hypothetical model of filipin III obtained by molecular modeling by using the absolute stereochemistry of the closely-related pentaene antibiotic pentamycin [14,15]. Oxygen atoms are represented as black spheres and hydrogen bonds are shown with dashed lines.

rotating injection syringe; the lipid suspension was then stored at 30°C.

2.3. Preparation of the antibiotic-lipid mixtures

Two types of mixtures were used. In the mixtures I, the desired amount of freshly-prepared DMSO solution of antibiotic was added to preformed MLV. In the mixtures II, the antibiotic and the phospholipid were dissolved in methanol and in chloroform, respectively, and the mixture was coevaporated under a nitrogen flow. The dried film was dispersed in the buffer and lyophilized; the resultant powder, redispersed in the buffer, was then vortexed and freeze-thawed several times through the gel-to-fluid transition temperature, T_m, to ensure homogeneity. In the mixtures II filipin is in contact with both sides of all the lamellas of one vesicle while in the mixtures I it is in contact only with the outer side of the external lamella.

The composition of mixtures was given by the antibiotic-to-phospholipid molar ratio, R.

2.4. Spectroscopic measurements

For UV absorption, a Cary 1E spectrophotometer, equipped with a thermostated cuvette holder, was used. The CD spectra were recorded with a Jobin Yvon Mark IV dichrograph, also equipped with a thermostated cuvette holder allowing to regulate temperatures with an accuracy of ± 0.2°C. $\Delta\varepsilon$ is the differential molar dichroic absorption coefficient. Spectra were corrected for light scattering by vesicles.

2.5. High-sensitivity differential scanning calorimetry

Experiments were performed on a DASM-4 microcalorimeter [12], interfaced to a Bull Micral computer for data storage. For analysis and integrations, the software

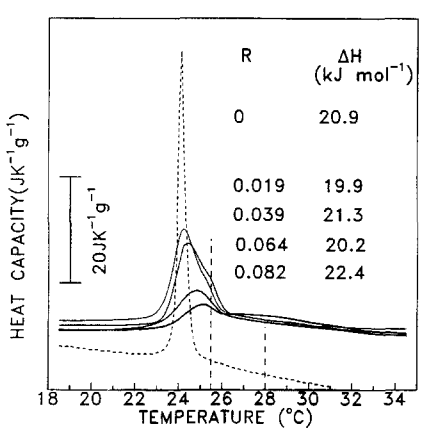

Fig. 2. Thermograms of pure DMPC (---) and DMPC-filipin mixtures II (————; sample weight: 3 mg; heating rate: 0.25 C°/min). The different filipin-to-DMPC molar ratios, R, from top to bottom correspond to endotherms in order of decreasing maxima. ΔH values were calculated by tracing linear baselines between the limit temperatures of the endotherms (cf. Methods).

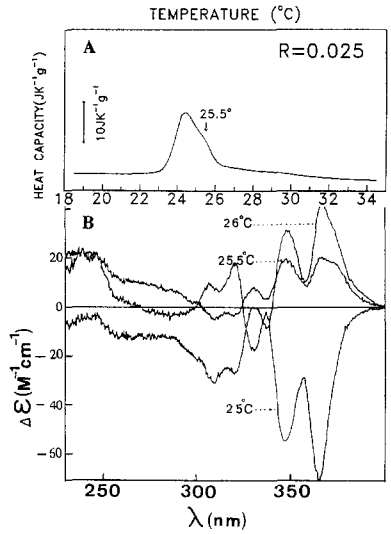

Fig. 3. Endotherm (A) and CD spectra within the endotherm temperature range (B) of a $R = 0.025$ DMPC-filipin mixture II. (cf. Methods).

ORIGIN from Microcal Inc. was used. The onset and the completion temperatures of the endotherms were defined as the intersections of their rising and falling edges with the initial and final baselines (which did not merge, generally). A straight line joining the two corresponding points on endotherms was used as the baseline for integrations.

2.6. Titration calorimetry

Titration experiments were carried out on a Omega titration calorimeter from Microcal Inc. A 3.5 mM lipid suspension was placed in a 250 μl injection syringe and a buffered 100 μM filipin solution, in the 1.34 ml sample cell. This inversion of the usual procedure was found the most satisfactory because the filipin solution had a large heat of dilution. Sequences of 40 successive 6-μl injections or 20 successive 12.5-μl injections were made, under stirring at 400 rpm. With the same sequence of injections, two blanks were performed: additions of the buffer to the filipin solution and of the lipid suspension to the buffer. During each experiment the temperature was regulated in a accuracy of ± 0.5°C. The area under each peak was calculated with the ORIGIN software provided by Microcal Inc.

2.7. Molecular modelling

A molecular model of the filipin III monomer was constructed in vacuo within the BUILDER module of the program INSIGHT II, from Biosym Technologies (San Diego, CA, USA).

3. Results

3.1. Structural aspect of filipin III

A modelling of the filipin III monomer (cf. Fig. 1) was made based on the covalent structure of filipin III [13] for

which no sterostructure or absolute stereochemistry is available. Since filipin III differs from pentamycin (= fungichromin, lagosin, cogomycin) by only one hydroxyl group which is replaced by one hydrogen at the C-13 position of filipin III, we assumed as reasonable to model filipin III with the same stereochemistry as that of pentamycin which has been recently established [14,15]. The proposed model appears more regular and more energetically favorable than the one recently proposed by Balakrishnan and Easwaran [16] which exhibits a reverse chirality at C-2 which is not experimentally supported by their NMR data and in discrepancy with the pentamycin stereostructure.

3.2. Influence of filipin on the thermotropic behaviour of DMPC bilayers

High-sensitivity DSC measurements were performed on diluted mixtures II ([DMPC] = 3.1 ± 0.2 mM), at a slow heating rate (0.25 C°/min). At least two successive scans were carried out; the good reproducibility indicated that those mixtures were in thermodynamical equilibrium.

As seen in Fig. 2, the onset temperature of the endotherms stayed constant at 22.7°C whatever the filipin content was ($R \leq 0.10$). In contrast, the higher the filipin content, the higher the completion temperature and the flatter the endotherm. This simultaneous extending and flattening resulted in a roughly constant global surface of the endotherms (enthalpy of 21 ± 2 kJ mol^{-1}, as reported in Fig. 2).

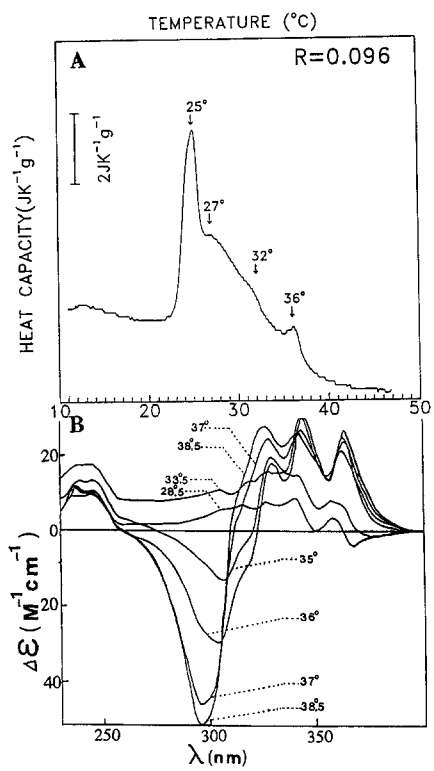

Fig. 4. Endotherm (A) and CD spectra within the endotherm temperature range (B) of a $R = 0.096$ DMPC-filipin mixture II (cf. Methods).

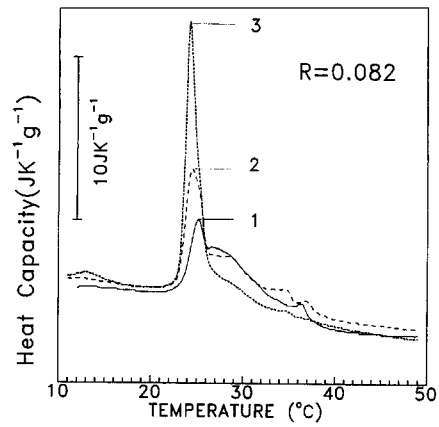

Fig. 5. Thermograms of a $R = 0.082$ mixture II (cf. Methods) obtained for three successive scans between 8 and 58°C, numbered in the successive order. The overall enthalpy remains equal to 22 ± 2 kJ mol^{-1}.

More or less sharp components, depending on the composition, appeared. As seen in Figs. 2 and 3A, a shoulder clearly appeared at 25.5°C for $0.019 < R < 0.039$. A bump around 28°C, was visible for $R = 0.082$ (cf. Fig. 2); and, for $R = 0.096$, two bumps and a small peak can be seen at 27, 32 and 36°C, respectively (cf. Fig. 4A). When the final temperature of the heating scan was beyond 55°C, these components tended to disappear from the subsequent scans, leading to an endotherm similar to the pure DMPC one. As seen on three successive scans till 58°C of a $R = 0.082$ mixture II (cf. Fig. 5), the peak, initially at 25.3°C, progressively increased in height and shifted to 24.1°C. The 25.5°C shoulder, and the bumps at 28 and 36°C disappeared, while the overall enthalpy remained equal to 22 ± 2 kJ mol^{-1}; at the third scan, the pretransition reappeared at 13°C. These results can be correlated with the modifications of the CD spectra of pure filipin aqueous solutions observed when the temperature exceeds 55°C (data not shown) which indicate a conformational change of filipin. By cycling the temperature between 8 and 58°C, filipin released from the DMPC bilayers during the heating scan has been conformationally modified so that it cannot be reincorporated during the cooling.

3.3. Influence of the DMPC bilayers on the filipin UV and CD spectra

The CD spectrum of pure filipin aqueous solutions depends on the concentration. Whereas at concentrations smaller than 20 μM the CD signal is very weak, over a concentration of 30 ± 10 μM, an intense excitonic doublet, with a negative peak at 281 nm, appeared (Fig. 6B; [17,8]). By analogy with the behaviour of other polyene antibiotics [18], such a doublet signalizes the self-association of filipin, due to its amphiphilic character. However, the threshold concentration obtained by us for this self-as-

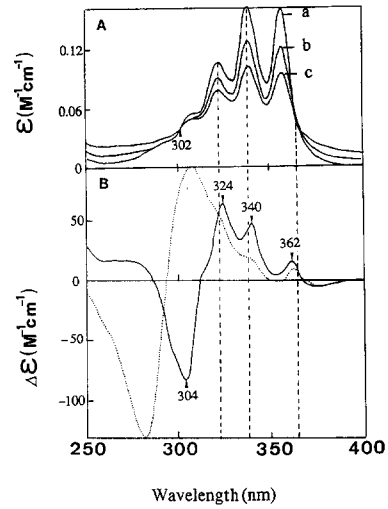

Fig. 6. Filipin spectra in the presence or not of fluid preformed DMPC MLV at 25.5°C. (A) Absorption spectra for DMPC/filipin = 0 (a), 2.1 (b), 4 (c) ([filipin] = 20 μM). (B) Dichroic spectra for DMPC/filipin = 0 (· · ·), 3.9 (————) ([filipin] = 90 μM).

sociation is at variance with recent results of Castanho et al. [19]. We think that this discrepancy arises from the different nature of used filipin (filipin complex instead of filipin III, cf. [9]); indeed, the molar absorption coefficient measured by these authors, in aqueous phase, is noticeably smaller than that determined by us ($\varepsilon_{340} = 4.7 \cdot 10^4$ M^{-1} cm^{-1}, cf. [20], instead of $8.0 \cdot 10^4$ M^{-1} cm^{-1}).

In the presence of DMPC bilayers, the filipin UV and CD spectra are modified. These modifications were followed only within the $T > T_m$ region since DSC results indicated that filipin interacts with DMPC bilayers only when they are in the fluid state.

Concerning CD spectra, the addition of preformed fluid DMPC MLV to a filipin aqueous solution (mixtures I), resulted at first in the disappearance of the filipin doublet and then its replacement by another, less intense, doublet with three positive peaks, at 324, 340 and 362 nm and a red-shifted negative peak, at 304 nm (cf. Fig. 6B). We attribute this qualitative change of the CD spectrum to the water-to-membrane transfer of filipin. This transfer can follow from a mere partitioning of filipin between the two phases or from an actual filipin-DMPC binding. In order to decide between these two possibilities, titrations of filipin aqueous solutions, by addition of DMPC MLV, were performed at different temperatures within the temperature range of endotherms. The amplitudes of any signal of the CD spectra (the positive peaks at 340 and 324 nm or the negative peak) exhibited a maximum for $(1/R) = 4.2 \pm 0.5$ ($1/R = 3.9$ at 25°C, 3.7 at 29°C and 4.7 at 35°C; cf. Fig. 7A). Such maxima establish the occurrence of a saturable binding of filipin to DMPC MLV (mole ratio method, cf. [21]); the constancy of the corresponding stoichiometry with the temperature indicates a great thermodynamic stability of the MLV-filipin association.

The specific CD spectrum of DMPC bound filipin enabled us to identify when filipin was in a bound state in mixtures II (cf. Methods). Paralleling the calorimetric measurements, the CD spectra of mixtures II studied by DSC were followed while increasing temperature from T_m, by increments of 0.5 or 1 C° (stepwise heating rate of around 0.17 C°/min). Whereas it was for a $R = 0.025$ mixture II that the 25.5°C shoulder on the thermogram was the most marked, the CD spectra of this mixture exhibited striking changes within 25–26°C (cf. Fig. 3B). Similarly, for a $R = 0.096$ mixture II, whereas the thermogram displayed a small peak at 36°C, the CD spectra exhibited the three positive peaks, characteristic of the bound filipin CD spectrum, exclusively at 35 and 36°C (cf. Fig. 4B). From 37°C, the negative peak became more intense and blue-shifted what signalized the release of filipin: this release occurred, as expected, at the completion temperature of the corresponding endotherm (37.5°C, cf. Fig. 4A).

In contrast with the CD spectra, the absorption spectra, changed little by adding DMPC MLV. The amplitudes of the bands of the vibronic progression characteristic of the pentaene chromophore, decreased and two isosbestic points at 302 and 365 nm appeared (cf. Fig. 6A).

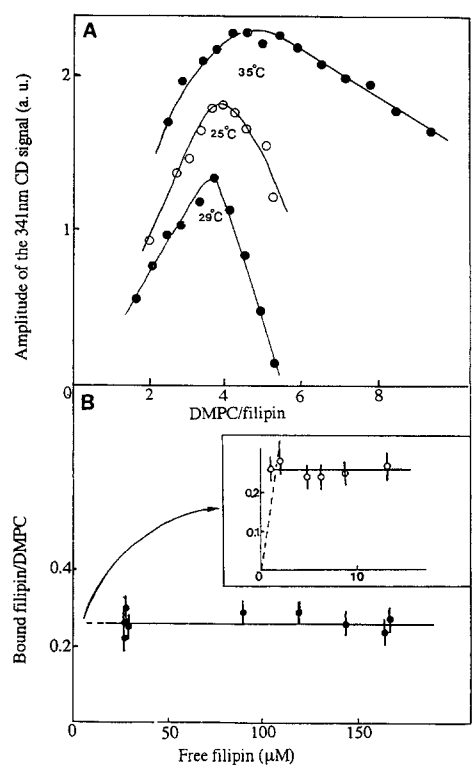

Fig. 7. Evidences for a strong saturable binding of filipin to DMPC MLV. (A) The amplitudes of the 341 nm CD signal as a function of the DMPC-to-filipin molar ratio, $1/R$, exhibit maxima at the same stoichiometry ($1/R = 4.2 \pm 0.4$) whatever is the temperature within the endotherm range. (B) On a direct plot of the binding isotherm [23] the asymptotic number of bound filipin molecule per DMPC is reached as soon as (free filipin) = 2 μM and agrees with the above-determined stoichiometry ($R = 0.26 = 1/3.8$).

3.4. Evaluation of the filipin-DMPC MLV association constant

The different characteristics of the free and bound filipin CD spectra enabled us to determine the respective concentrations of each species. Such determinations were performed at 25.5°C either by adding MLV to an excess amount of filipin or by adding filipin to an excess amount of MLV. When the filipin concentrations were lower than 30 μM (CD signal very weak) we made use of the modifications of the filipin UV spectra in the presence of DMPC MLV to determine the bound filipin concentrations, C_B. Using Bittman's method [22] and referring to the 339 nm absorbance in the presence of DMPC MLV, A, C_B was deduced from:

$$C_B = \frac{\varepsilon_F C_T - A}{\varepsilon_F - \varepsilon_B}$$

where C_T is the total filipin concentration and ε_F and ε_B the 339 nm molar absorption coefficients of the free and bound filipin, separately determined ($\varepsilon_F = 8.0\ 10^4$ $M^{-1}cm^{-1}$ and $\varepsilon_B = 4.9\ 10^4$ $M^{-1}cm^{-1}$). The C_B values, at 25.5°C, were plotted (cf. Fig. 7B) with those obtained by CD as a 'direct plot' of the binding isotherm [23]. The experimental points were located around a horizontal at 0.26 ± 0.04. Such an observation is relevant. Generally speaking, when a ligand L (the antibiotic) binds to a substrate S (the phospholipid bilayer), which has n independent fixation sites per molecule, the average number of bound ligand molecules per molecule of substrate has the following expression [23]:

$$\bar{i} = \frac{\sum_{i=1}^{n} i[SL_i]}{[S_{tot}]} = \frac{nk[L]}{1 + k[L]}$$

where [L], $[S_{tot}]$ and $[SL_i]$ are the concentrations of the free ligand, the total substrate and the association of i molecules of ligand with one molecule of substrate, respectively; k is the microscopic association constant corresponding to each independent fixation site. The corresponding binding isotherm shows an hyperbolic dependence on the free ligand concentration with an asymptote equal to n and approaches a linear function, with the slope nk, when [L] is so small that $k[L] \ll 1$. In our case, the asymptote was reached as soon as the free filipin concentration was 2 μM (cf. inset of Fig. 7B). The corresponding number of bound filipin per DMPC (0.26 ± 0.04, cf. Fig. 7B) agrees fairly with the inverse of the DMPC-to-filipin molar ratio at saturation, independently determined ($1/R = 4.2 \pm 0.5$, cf. Fig. 7A) From the smallest filipin free concentration, for which this asymptote was reached, the k value could be evaluated. The slope at the origin is $nk \geq 1.5 \cdot 10^5$ M^{-1} (cf. inset of Fig. 7B). Therefore, k is larger than $5.8 \cdot 10^5$ M^{-1}: everything happens as if the filipin ligand has four independent fixation sites with the same association constant $k \geq 6 \cdot 10^5$ M^{-1}.

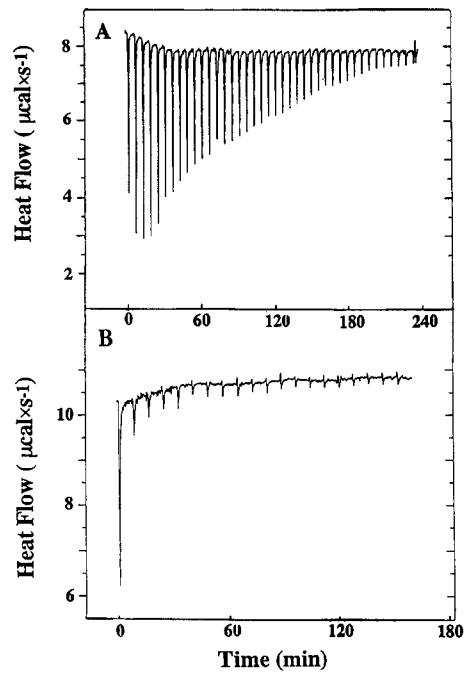

Fig. 8. Row data of typical isothermal titration calorimetry experiments at two temperatures. A 100 μM buffered filipin solution in the 1.34 ml reaction cell is titrated by a 3.5 mM suspension of DMPC MLV. (A) 40 successive 6-μl injections at 6-min intervals at 30°C. (B) 20 successive 12.5-μl injections at 8-min intervals at 40°C. The heat release becomes negligible from the 35th injection (DMPC/filipin = 5) at 30°C and from the 2nd one (DMPC/filipin = 0.7) at 40°C.

3.5. Enthalpy change associated with the water-to-membrane transfer of filipin

Titration calorimetry measurements were performed at 21, 26, 30, 35 and 40°C. Aliquots of a DMPC MLV suspension were added to a buffered filipin solution (cf. Methods). After correction for the blanks (cf. Methods), the heat released during the filipin transfer from water to DMPC membranes was obtained as a function of the μmole number of DMPC, as shown in Fig. 10. It is noteworthy that, whereas the transfer was exothermal at 26, 30 and 35°C, i.e. within the temperature range of DSC endotherms, the heat effect at 21 and 40°C was negligible (cf. Fig. 8B). Moreover, let us stress that, at 30°C, the heat release becomes negligible from a DMPC/filipin molar ratio equal to 5 (cf. Fig. 8A), in reasonable agreement with the stoichiometry at saturation determined by CD.

4. Discussion

4.1. Information given by methods focused on the state of the antibiotic

The addition of fluid DMPC MLV to filipin aqueous solutions, promotes a change of the antibiotic conformation, as detected by CD (cf. Fig. 6B). The drastic lowering

of the CD signal before the appearance of a new spectrum, suggests that filipin is partitioned between aqueous and membranous phases under its monomeric form, whose CD signal is very weak; subsequently, it self-associates inside the membranes, as indicated by the presence of a new excitonic doublet.

It is interesting to compare our results with those from Schwarz et al. [24], on the incorporation of the peptide alamethicin into dioleoylphosphatidylcholine SUV, followed by CD. When adding SUV to an alamethicin solution, the amplitude of the CD signal, reached a plateau; plateau values increased with the alamethicin concentration. They concluded that the incorporation of the peptide into the membranes is cooperative and unsaturable. On the contrary, in our case, appears a saturation which is probably reached by a quasi step-function (cf. inset of Fig. 7B). So, while in Schwarz et al. experiments the process is a mere partitioning of the solute (alamethicin) between the aqueous and the membranous phases, in our case it could be viewed as an actual binding of filipin to saturable fixation sites.

In the light of the CD spectra the components observed at certain temperatures different from T_m on the DSC recordings of mixtures II (cf. Figs. 2 and 3A and Fig. 4A) can be interpreted. In Figs. 3 and 4, the evolutions with the temperature of the CD spectra and the corresponding thermograms, for the $R = 0.025$ and 0.096 mixtures II were compared. The characteristic bound filipin spectrum, with its three positive peaks at 324, 340 and 362 nm, exclusively appears at 25.5°C for $R = 0.025$ (Fig. 3B) and at 35 and 36°C for $R = 0.096$ (Fig. 4B). It is just the temperatures for which the corresponding endotherms exhibit a marked component: a shoulder at 25.5°C for $R = 0.025$ (Fig. 3A) and a small peak at 36°C for $R = 0.096$ (Fig. 4A). Now, it is worth pointing out that, in mixtures of non-isomorphous molecules A and B, like ours, it can be present, in addition to B-rich and B-poor phases, some compound-like phases with intermediate compositions [25]. Therefore, it is tempting to suppose that these components reflect the emergence of compound-like phases as the most stable phase, giving rise to invariant regions of the phase diagram (fixed temperature and composition). Nevertheless, a confirmation of this interpretation would require a more extensive study.

4.2. Information given by methods focused on the state of the bilayers

When increasing the filipin content the endothermic zone of thermograms becomes asymmetric with a onset temperature unchanged (22.7°C, in good agreement with the well-documented onset of the pure DMPC gel-fluid transition, cf. [26]) while the high-temperature limit increases. This result amounts to that on a phase diagram the high-temperature frontier of the gel phase region (the solidus line) is independent on the composition of the mixtures: filipin is not miscible with gel DMPC bilayers. This inference is confirmed by the titration calorimetry experiments. Whereas the water-to-membrane transfer of filipin is exothermal by a few tens of kJ mol^{-1}, at 26°C ($> T_m$), it becomes athermal at 21°C ($< T_m$); that indicates that the transfer does not occur when the DMPC MLV are in gel phase.

Moreover, it should be recalled the previous result obtained by ^{2}H-NMR for mixtures of perdeuteriated DMPC MLV with filipin. When the filipin-to-DMPC molar ratio was higher than 0.12, the fluid phospholipid matrix displayed some domains exhibiting the dynamic behaviour of

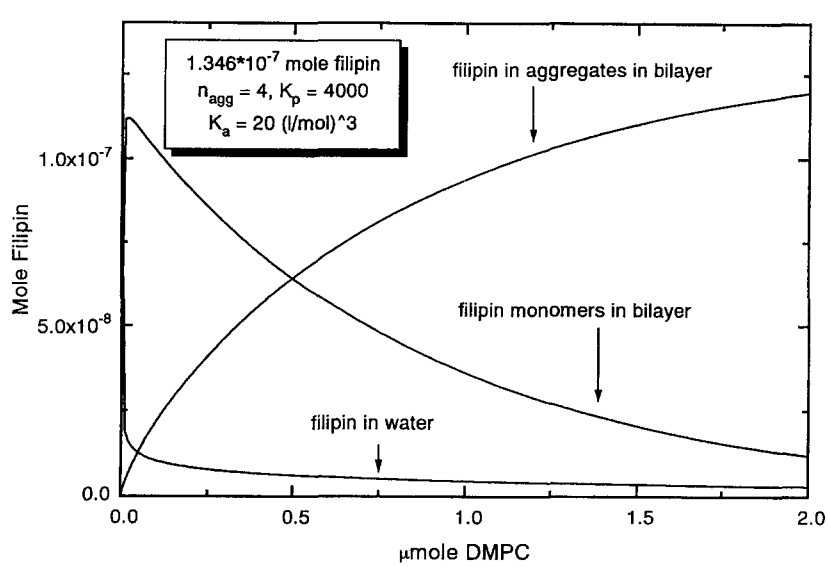

Fig. 9. Calculated number of moles of filipin in water and in DMPC bilayers as a function of added DMPC, adapted to the specific experimental situation of the calorimetric experiment represented in Fig. 8A. n_{agg} = aggregation number, K_p = partition coefficient, K_a = aggregation equilibrium constant of filipin (see text).

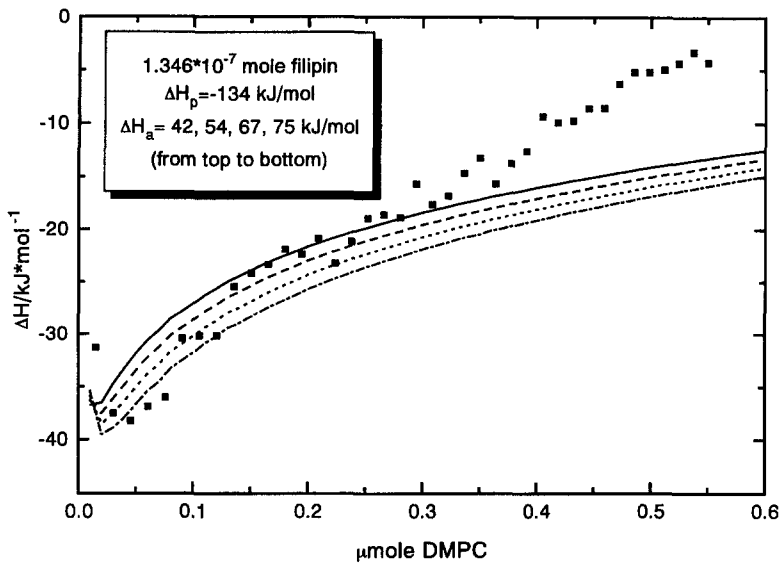

Fig. 10. Calorimetric titration curves calculated from the curves shown in Fig. 9. ΔH_p = transfer enthalpy of filipin from water to the bilayer, ΔH_a = aggregation enthalpy of filipin. ■, experimental values.

gel lipids, between T_m and $T_m + 11°C$ [8]; these domains were attributed to filipin-DMPC complexes. This result associated with the change of the filipin conformation in the presence of DMPC MLV argue for an incorporation of filipin into the hydrophobic core of the fluid DMPC bilayers in contact with the lipid chains.

4.3. Thermodynamics of the water-to-membrane transfer of filipin

In order to interpret the titration calorimetry results the isothermal titration curves, like this shown in Fig. 10 (squares), were simulated by assuming a simple partitioning of the antibiotic between water phase and membrane. In addition, it was assumed that the antibiotic molecules aggregate in the bilayer whereas their aggregation in water was neglected. Even in this simple model, there are five parameters which have to be varied to fit the experimental curves: the partition coefficient, $K_p = C_m/C_w$, of the antibiotic between membrane and water (C_m = concentration of filipin in membrane, C_w = concentration of filipin in water), the aggregation number, n, of the antibiotic molecules in membrane, the aggregation equilibrium constant, $K_a = C_a/C_m$ (C_a = concentration of filipin aggregates in membrane), the enthalpy of the water-to-membrane transfer of the antibiotic, ΔH_p, and the aggregation enthalpy, ΔH_a. In this model, no specific binding of lipid molecules to the antibiotic is assumed; an inclusion of a multiple-step binding to independent binding sites, would be possible but would increase the number of free parameters and would complicate the calculations. Fig. 9 shows a plot of the number of moles of free filipin in water, monomeric and aggregated filipin in the membrane, as a function of added DMPC. The plot constructed from the model described above was calculated for the specific situation of the titration experiment shown in Fig. 8A. From these curves the calorimetric titration curve can be calculated, with ΔH_p and ΔH_a as parameters, as shown in Fig. 10 (full line). The beginning of the titration curve is adequately described by the model, but, at higher dilution of filipin in the bilayer, the calculated curves deviate significantly from the experimental values. In all cases, ΔH_p is negative and ΔH_a positive. This is quite reasonable when the DSC data are taken into account. Thus, the exothermic value of ΔH_p may mainly be caused by an ordering of the 'fluid' DMPC, in compliance with the extent of the endotherms towards $T > T_m$ and with our previous ^{2}H-NMR results [8]. Generally speaking, the enthalpy of transfer of a hydrophobic solute from water to an apolar medium depends on the temperature. For the transfer of a hydrocarbon from water to its neat liquid phase, it changes from a positive to a negative value when increasing temperature from 25 to 50°C [27]. Similar results have been obtained for the transfer of amphiphilic molecules from water to a micelle [28]. However, the latter value, at 30°C (between −8 and +8 kJ mol^{-1}) is much smaller than our experimental ΔH value (−38 kJ mol^{-1}, cf. Fig. 10) and our calculated ΔH_p value (−84 to −125 kJ mol^{-1}). This points to the ordering of the DMPC chains which will be discussed elsewhere. Taking into account the stoichiometric ratio of 4 DMPC molecules per filipin, the calculated ΔH_p value corresponds to −21 to −29 kJ per mole of DMPC, approximately the negative melting enthalpy of pure DMPC. However, monomeric filipin in a bilayer would be surrounded by more DMPC molecules per filipin than the aggregate, so that the enthalpy per DMPC molecule gets lower, which is quite reasonable. The positive ΔH_a value for aggregation is easy to understand in the frame work of this model. Dissociation of filipin aggregates in the bilayer into monomers is an

exothermic process, as more DMPC molecules are affected, i.e. 'ordered', by monomeric filipin than by the filipin aggregate. The ΔH_a value of 42 to 50 kJ per mole of filipin can then be accounted for by an increase of 2–3 molecules of DMPC per 'solvated' DMPC.

Assuming a hexagonal arrangement of molecules, with an area for the filipin molecule similar to that of DMPC, a monomeric filipin would be surrounded by 6 DMPC molecules and an aggregate of 4 filipin molecules by 10 DMPC molecules, i.e. 2.5 DMPC/filipin. Taking a value of -17 kJ per mole of DMPC as the ordering enthalpy, one then estimates for ΔH_p a value of -100 kJ per mole of filipin and for $\Delta H_a + 58$ kJ per mole of filipin, values well within the range of our calculated values. The poor agreement of the calculated curves at high lipid/filipin ratios is, of course, a result of the simplicity of our model. We simulated these curves with fixed values for the partition coefficient K_p and the partitioning enthalpy ΔH_p. This implies ideal mixing behaviour of filipin with DMPC. This is highly unlikely due to the different shapes of the two molecules. Therefore, assuming K_p and ΔH_p values which are dependent on mixing ratio, into the model, would probably give better fits. However, at the present stage the amount and precision of experimental data does not warrant a more complicated model.

Now, let us come round to a recent debate concerning the soundness of approximating a membrane to a mere apolar solvent, from a thermodynamical viewpoint. Results obtained since a few years on the energetics of the transfer of amphiphilic solutes from water to a membranous phase, at room temperature [29,30], provide evidences against such an approximation. Indeed, in the free energy expression, $\Delta G = \Delta H - T\Delta S$, for the transfer of such solutes from water to an apolar medium, the driving term is the entropic one when this medium is an organic solvent ($-T\Delta S < 0$, favorable); it reflects the entropic character of the hydrophobic effect interpreted as the release of the ordered hydration shell surrounding the hydrophobic residues of the solute in water. On the contrary, for the transfer to membranes, the driving term is quite often the enthalpic one ($\Delta H < 0$, favorable). This contradiction has been recently clarified by comparing directly the energetics of the transfers of a solute to either an organic solvent or a lipid bilayer [31]. In both cases, a noticeable decrease of the heat capacity appears, proving the occurrence of the hydrophobic effect. However, the respective contributions of the entropic and the enthalpic terms, especially the latter, were different. Such a difference has been assigned to a complementary thermal effect ('bilayer effect'), associated with a reorganization of the bilayer subsequent to the incorporation of the solute, modifying the energetics of the hydrophobic effect. Our results confirm this viewpoint. A clear decrease of the heat capacity during the partitioning of filipin, identifying the hydrophobic effect, appears on the endotherms (cf. Fig. 4A). On the other hand, from our simulation of our titration calorimetry measurements, the partitioning coefficient, K_p, is about 4000 which leads to a value of about -21 kJ mol^{-1} for $\Delta G_p = -RT \ln K_p$. As the ΔH_p value derived from our simulation is more negative, it can be concluded that the $-T\Delta S_p$ term is positive (i.e. $\Delta S_p < 0$, unfavorable): the partitioning is enthalpy-driven. These negative ΔS_p and ΔH_p represent the thermal effects expected from our ^{2}H-NMR observations [8], namely a partial ordering of the DMPC lipid chains, associated, by nature, with negative entropy and enthalpy changes. Similar results were obtained with another polyene antibiotic (nystatin) whose incorporation into dilauroylphosphatidylcholine bilayers was followed by DSC and ^{2}H-NMR (unpublished results). In this case, the ordering of the lipid chains, promoted by the antibiotic occurred on the entire lipid chains. These results prompt us to conclude that the reorganization of the bilayers provoked by the incorporation of the polyene antibiotics (the 'bilayer effect' invoked by Wimley and White) is an ordering of the lipid chains. In a molecular description, this ordering suggests that the antibiotic inside the membranes is located in such a way that the plane of the rigid polyenic chain is parallel to the lipid chains thereby inducing an ordered all-*trans* configuration of the latter close to the antibiotic

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