ELSEVIER

Biochimica et Biophysica Acta 1372 (1998) 227–236

BIOCHIMICA ET BIOPHYSICA ACTA
BBA

Proton induced vesicle fusion and the isothermal $L_\alpha \rightarrow H_{II}$ phase transition of lipid bilayers: a ^{31}P-NMR and titration calorimetry study

Markus R. Wenk [1], Joachim Seelig *

Department of Biophysical Chemistry, Biocenter of the University of Basel, Klingelbergstr. 70, CH-4056 Basel, Switzerland

Received 7 January 1998; revised 26 March 1998; accepted 1 April 1998

Abstract

The proton-induced isothermal fusion of *uni*lamellar lipid vesicles (Duzgunes et al., Biochemistry 24 (1985) 3091–3098) is compared with the lamellar (L_α) → hexagonal (H_{II}) phase transition of *multi*lamellar lipid dispersions. Both lipid systems are composed of 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoethanolamine (POPE) and oleic acid (OA) at a 7:3 molar ratio. Using solid-state phosphorus-31 nuclear magnetic resonance (^{31}P-NMR) it is demonstrated that the multilamellar lipid dispersions are in the bilayer state at physiological pH and undergo a $L_\alpha \rightarrow H_{II}$ phase transition between pH 6.3 and 5.7. This phase transition can also be induced at constant pH by increasing the temperature. The midpoint of the temperature-induced $L_\alpha \rightarrow H_{II}$ transition is $T_h = 56$°C (at pH 7.4) and the corresponding transition enthalpy is $\Delta H = 0.7 \pm 0.1$ kcal/mol as determined with differential scanning calorimetry. Both the proton-induced and the temperature-induced phase transition can be completely inhibited by addition of 30 mol% of 1-palmitoyl-2-hydroxy-*sn*-glycero-3-phosphocholine (LPC). In a second set of experiments unilamellar vesicles are prepared either by sonication or by extrusion through polycarbonate filters at pH 7.4 and are titrated into buffer at pH 5.7. The proton-induced fusion of the lipid vesicles is monitored with isothermal titration calorimetry, light scattering and fluorescence spectroscopy. The fusion reaction is characterized by an endothermic enthalpy of $\Delta H = 0.5 \pm 0.2$ kcal/mol (at 28°C). The fusion enthalpy is independent of the vesicle diameter and is only slightly reduced by an increase in temperature to 50°C. Vesicle fusion is accompanied by an increase in light scattering, indicating the formation of larger lipid structures. The transition from unilamellar vesicles to fused lipid structures occurs in the same narrow pH range of 6.3–5.7 as observed for the $L_\alpha \rightarrow H_{II}$ transition of multilamellar dispersions. Vesicle fusion can be inhibited with 30% LPC. The virtually identical set of parameters found for the $L_\alpha \rightarrow H_{II}$ phase transition and the vesicle fusion reaction suggests that vesicle fusion also entails a $L_\alpha \rightarrow H_{II}$ phase transition.

Keywords: Lipid phase transition; Hexagonal phase; Phosphatidylethanolamine; Isothermal titration calorimetry; Lysophosphatidylcholine; ^{31}P-NMR

1. Introduction

Non-bilayer arrangements of phospholipids, such as the inverted hexagonal (H_{II}) phase and the inverted cubic (I_{II}) phase can be formed under a variety of conditions. They are believed to play an important role as transient structures in complex

* Corresponding author. Fax: +41 (61) 267-2189; E-mail: seelig1@ubaclu.unibas.ch

[1] Present address: Yale University School of Medicine, Department of Cell Biology, New Haven, CT 06510, USA.

0005-2736/98/$19.00
PII: S0005-2736(98)00059-5

biological processes such as membrane fusion (for a recent review cf. [1]) and are also implied as structural features in tight junctions [2]. Non-bilayer structures and the conditions needed for their formation have therefore been the subject of a large body of both experimental and theoretical work [3–7]. The lamellar (L_α) → hexagonal (H_{II}) phase transition has been investigated experimentally as a function of temperature, degree of hydration, and salt concentration using X-ray diffraction, electron microscopy and ^{31}P-NMR [8–10]. Differential scanning calorimetry was used to determine the transition temperature and the enthalpy of the thermotropic $L_\alpha \rightarrow H_{II}$ reaction for a variety of lipid mixtures [11–14]. A theoretical concept consistent with the experimental observations is the 'shape model' originally proposed by Israelachvili and coworkers [15,16]. According to this model the lipid molecules are considered as building blocks of different shapes, i.e. they are assumed to adopt either cone-shaped, cylindrical or inverted-cone shaped structures. Depending on the experimental conditions and the membrane composition the interplay of different geometries leads to the formation of micelles, bilayers, or hexagonal phases, respectively. The best studied examples of non-bilayer lipids are phosphatidylethanolamines (PE) which exhibit a high tendency to form the inverted hexagonal (H_{II}) phase. Phosphatidylethanolamines are common constituents of biological membranes and are thought to play an essential role in the formation of non-bilayer intermediates necessary for cell fusion and fission. Possible geometries of such intermediate structures in the transition pathways have been proposed and the free energy change involved in their formation has been estimated theoretically [3,4,17,18].

The great majority of experimental data relates to a thermally induced $L_\alpha \rightarrow H_{II}$ phase transition. Here we use a different approach and induce the $L_\alpha \rightarrow H_{II}$ transition by a pH change. The studies are performed with either multilamellar phospholipid dispersions or unilamellar lipid vesicles composed of 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoethanolamine (POPE) in mixture with oleic acid (OA), typically at a mixing ratio of POPE/OA = 7:3 (mol/mol). The phase behavior of the multilamellar dispersions is monitored with solid-state phosphorus-31 nuclear magnetic resonance (^{31}P-NMR) as a function of pH and temperature. Unilamellar vesicles prepared by sonication of the same lipid mixture are stable at physiological pH but aggregate and fuse upon lowering the pH to 5.7 [19]. We measure the fusion process with high sensitivity titration calorimetry by injecting POPE/OA vesicles into low pH buffer and compare the thermodynamic parameters of the fusion reaction with those of the $L_\alpha \rightarrow H_{II}$ transition. The data provide new molecular insight into the fusion reaction.

2. Materials and methods

1-Palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoethanolamine (POPE) was a product of Avanti Polar Lipids (Birmingham, AL, USA). 1-Palmitoyl-2-hydroxy-*sn*-glycero-3-phosphocholine (LPC) and oleic acid (OA) were purchased from Fluka with purities $>99\%$ (TLC). All chemicals were used without further purification. Buffers were prepared from 18 MΩ water obtained from a Nanopure A filtration system.

2.1. Preparation of vesicles

POPE and oleic acid were mixed as chloroform solutions to yield a POPE/OA ratio of 7:3 (mol/mol) and dried under a stream of nitrogen, followed by high vacuum for 1 h in the dark. Dichloromethane (0.3 ml) was added and again evaporated under nitrogen. High vacuum was applied overnight. The lipid film was hydrated in a defined amount of buffer (10 mM Tris, pH 7.4; 100 mM NaCl, lipid concentration $C_L \approx 2$ mg/ml) and the sample vortexed vigorously for 2–4 min.

Small unilamellar vesicles (SUV) were prepared by sonication (4°C) for 20–30 min until the solution became transparent. The sample was centrifuged for 8 min in an Eppendorf table top centrifuge at high speed (14 000 rpm) to remove metal debris and large particles. The supernatant had a slightly opalescence appearance and was stored at 4°C in the dark until use.

Large unilamellar vesicles (LUV) of diameter $d \approx 400$, 100 and 50 nm were obtained by extrusion of multilamellar lipid suspensions through polycarbonate filters (Nuclepore, Pleasanton, CA) [20,21]. The dried lipid was suspended in buffer, vortexed,

and freeze–thawed for five cycles. Unilamellar vesicles of the desired diameter were extruded under nitrogen pressure through filters by stepwise decreasing the pore size ($d = 400$ nm, $d = 200$ nm, $d = 100$ nm, and $d = 50$ nm, five times for each filter).

Lipid concentrations were determined gravimetrically by carefully weighing the samples and by adding defined amounts of buffer.

2.2. High sensitivity isothermal titration calorimetry

High sensitivity titration experiments were performed with a MicroCal Omega isothermal titration calorimeter (Microcal, Northampton, MA, USA) as described previously [22]. To avoid air bubbles, solutions were degassed under vacuum prior to use. The calorimeter was calibrated electrically. The data were acquired by computer software developed by MicroCal. Data was collected and processed with the software package 'Origin' developed by MicroCal.

2.3. Differential scanning calorimetry

Differential scanning calorimetry experiments were made with a Microcal MC-2 scanning calorimeter (Microcal, Northampton, MA, USA). POPE and oleic acid were mixed in chloroform and dried under a stream of nitrogen. High vacuum was applied overnight. A defined amount of buffer was added to the lipid film to yield lipid concentrations of $C_L \sim 5$ mg/ml. The suspension was vortexed extensively and subjected to several freeze–thaw cycles. After degassing, the samples were loaded into the calorimeter cell. The reference cell contained buffer. Heating scans were performed at a scan rate of 45 K/h. Data was collected and analyzed by the software package 'Origin' developed by MicroCal.

2.4. NMR measurements

Solid-state ^{31}P-NMR measurements of membranes were recorded on a Bruker MSL 400 NMR spectrometer operating at a phosphorus-31 frequency of 161 MHz. A spin echo sequence with gated proton-decoupling was used. The recycling delay was 5 s, the $\pi/2$ pulse width 3.05 μs and the interpulse spacing 40 μs. Between 8000 and 12000 free induction decays were accumulated. Usually ~ 10 mg of lipid (POPE and OA with or without LPC) were mixed in organic solvent. The solvent was evaporated with a stream of nitrogen and buffer of different pH (~ 5 ml) was added. The suspension was vortexed extensively, and subjected to ~ 10 freeze–thaw cycles. Next the lipid suspension was centrifuged at $400\,000 \times g$ for 30 min (4°C) in a Beckman TL-100 ultracentrifuge and ^{31}P solid-state nuclear magnetic resonance spectra were recorded at 301 K from the pellets.

3. Results

3.1. ^{31}P-NMR measurements of multilamellar lipid dispersions

The $L_\alpha \rightarrow H_{II}$ transition of PE containing lipid mixtures has been investigated in detail in the past. In most of these studies the transition was induced by increasing the temperature or changing the degree of hydration [8,23,24]. In the present work, the transition is induced by a moderate acidification of the lipid mixture at room temperature.

Fig. 1A shows a ^{31}P-NMR spectrum of a lipid dispersion composed of 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoethanolamine (POPE) and oleic acid (OA) (7:3 molar ratio) at pH 7.4 and 28°C. The asymmetric spectrum with a low field shoulder and a high field peak, separated by $\Delta\sigma = -38.0$ ppm, is characteristic of a lipid bilayer [25,26]. Fig. 1C shows the same lipid mixture at pH 5.7 (28°C). The spectrum is reversed in sign and narrowed by a factor of 2. This type of spectrum is typical for a hexagonal arrangement of the lipid molecules [26,27]. Fig. 1B was recorded at an intermediate pH of 6.2 and the spectrum corresponds to a superposition of Fig. 1A and C. Composite spectra like that shown in Fig. 1B were found in a rather narrow pH range of $5.7 \leq 6.3$. Fig. 1A–C provides evidence for a proton-induced lipid phase transition from the lamellar (L_α) to a hexagonal phase, most probably the H_{II} phase, at a constant temperature of $T = 28$°C. The midpoint of the transition is at pH ~ 6.0.

Lysophosphatidylcholines have been shown to inhibit the formation of hexagonal phases. This bilayer stabilizing effect of lysophosphatidylcholines on model lipid bilayers has been demonstrated recently by ^{31}P-NMR [28,29]. Fig. 1D then confirms the bi-

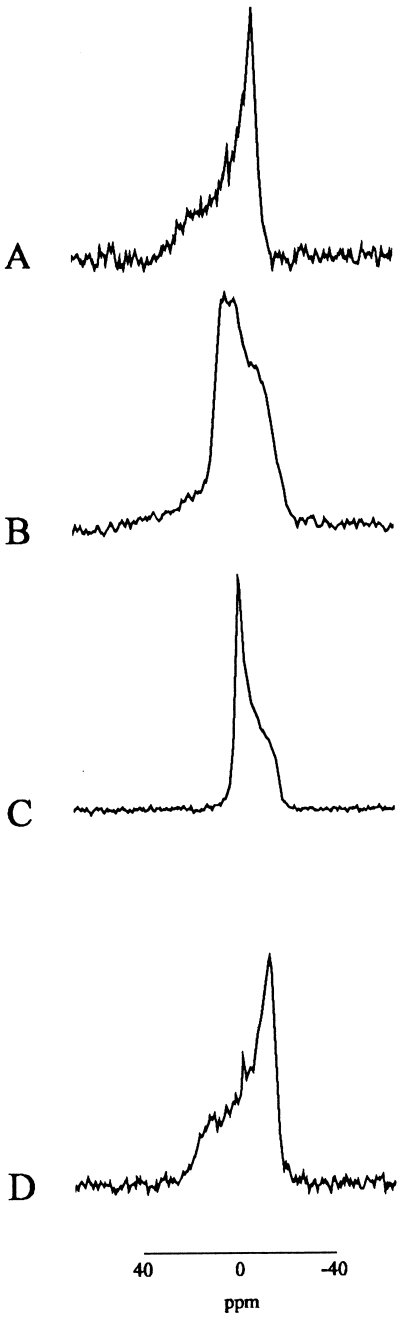

Fig. 1. ^{31}P-NMR spectroscopy of multilamellar lipid dispersions composed of POPE, oleic acid and lysophosphatidylcholine. Proton-induced $L_\alpha \rightarrow H_{II}$ transition at varying pH. Samples were prepared as described in Section 2. (A) POPE/OA (7:3, mol/mol) in 10 mM Tris, pH 7.4, chemical shielding anisotropy $\Delta\sigma = -38.0$ ppm. (B) POPE/OA (7:3) in 10 mM MES, pH 6.2. (C) POPE/OA (7:3) in 10 mM MES, pH 5.7, $\Delta\sigma = +19.6$ ppm. (D) LPC/POPE/OA (2:5:2) in 10 mM MES, pH 5.7, $\Delta\sigma = -35.1$ ppm. The ^{31}P chemical shielding anisotropy ($\Delta\sigma$) was evaluated from the separation between the low-field and the high-field shoulder at half-height of the signal intensity. $T = 28$°C.

layer stabilizing properties of LPC also for the present lipid mixture of POPE and oleic acid. Spectrum 1D arises from a ternary mixture containing POPE, oleic acid, and, in addition, 1-palmitoyl-2-hydroxy-*sn*-glycero-3-phosphocholine (LPC) (POPE/OA/LPC = 5:2:2, mol/mol/mol) measured at a low pH of 5.7. The spectrum is again typical for a lipid bilayer. It demonstrates that lysophosphatidylcholine inhibits the pH-induced formation of the hexagonal phase and stabilizes the POPE/OA bilayer at low pH.

3.2. *Fusion of unilamellar vesicles studied with titration calorimetry*

Lysophosphatidylcholines have also been shown to inhibit the *fusion* of both artificial and biological membranes [30–34]. We have taken advantage of this phenomenon to investigate the thermodynamics of the proton-induced fusion of lipid vesicles with high sensitivity titration calorimetry. Vesicles composed of POPE and oleic acid were prepared at pH 7.4. These lipid suspensions were injected into a calorimeter cell containing low pH buffer to induce the fusion reaction [19]. As a control, vesicles were incubated with LPC prior to acidification. These bilayer vesicles have lost the ability to fuse when titrated into low pH buffer and were used to correct for the enthalpy of buffer protonation.

Fig. 2A (solid line) shows three consecutive injections (10 μl) of sonicated unilamellar vesicles (POPE/OA (7:3), $C_L = 4.94$ mM, pH 7.4) into the reaction cell ($V = 1.3353$ ml) which contained buffer at pH 5.7. The reaction is fast and finished within the time resolution of the calorimeter. The reaction enthalpy per injection is obtained by integration of the area underneath the calorimeter tracing and is displayed in Fig. 2B (closed circles). It is constant for all three injections and amounts to $h = -32.7$ μcal (Fig. 2B and Table 1). In the control experiment, the lipid suspension was incubated with LPC (added from an aqueous stock, LPC/POPE/OA = 1:5:2, mol/mol/mol) prior to titration. As can be seen from Fig. 2A (dashed line) titration of these liposomes into

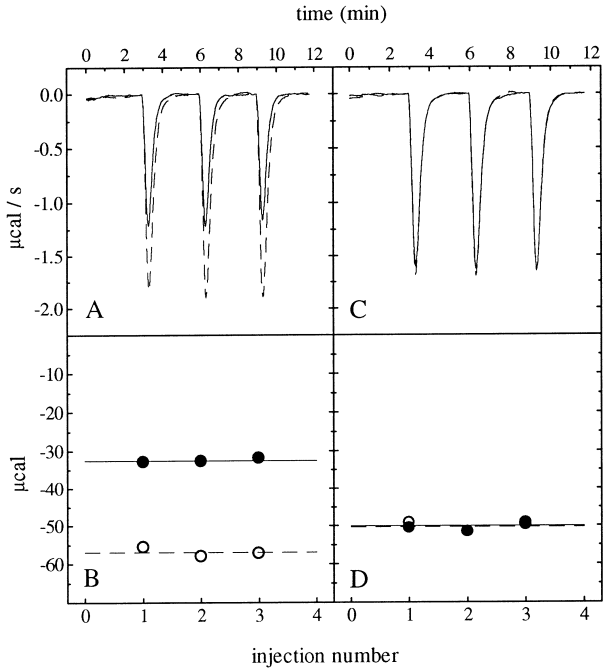

Fig. 2. Titration calorimetry of unilamellar vesicles of POPE and OA. Proton-induced fusion and inhibition by LPC. (A) 10 µl of sonified lipid vesicles (POPE/OA (70:30, mol/mol), 4.94 mM lipid concentration in 10 mM Tris, pH 7.4; 100 mM NaCl) were injected into the reaction cell ($V = 1.3353$ ml) which contained low pH buffer (10 mM MES, pH 5.7; 100 mM NaCl, $T = 28°C$) (solid line). The experiment with LPC-treated liposomes (LPC/lipid = 1:7, mol/mol, $C_L = 4.94$ mM) is shown as a broken line. The reference cell contained 10 mM MES, pH 5.7; 100 mM NaCl. Negative peaks represent exothermic reactions. (B) Heats of reaction obtained by integration of the area under the calorimeter tracings. Data from LPC treated and untreated liposomes is shown as open and closed circles, respectively. The solid and the dashed lines are the mean values of the heats of reactions of the untreated and the LPC-treated liposomes, respectively. (C,D) As (A) and (B) except that the reaction (and the reference) cell contained buffer at pH 6.3 (non-fusogenic pH).

low pH produces a distinctly more exothermic reaction heat of $h_c = -57.0$ µcal (Fig. 2B (open circles) and Table 1). In the absence of LPC the lipid vesicles fuse [19], in the presence of LPC the vesicles remain unaltered. Hence, the difference of the two reaction enthalpies yields the heat of the fusion reaction as $\delta h = h - h_c = -32.7 - (-57)$ µcal $= +24.3$ µcal. In each injection $n_L = 49.4$ nmol of lipid (POPE+OA) were injected. The molar heat of reaction therefore is endothermic with $\Delta H = \delta h / n_L = +490$ cal/mol. The experiment was repeated at different lipid concentrations. The values of h and h_c varied with the lipid concentrations, but the molar heat of reaction was virtually unchanged (Table 1). The influence of the vesicle size on the reaction enthalpy was also investigated by using extruded vesicles of different diameter. ΔH was found to be independent of the vesicles size with $\Delta H = +0.5 \pm 0.1$ kcal/mol in all cases (Table 1).

In the above experiments, the pH jump was large enough (ΔpH = 1.7) to induce complete fusion. Next the pH in the reaction cell was decreased in small steps from pH 7.4 while the injected vesicles were left at pH 7.4. Fusogenic vesicles without LPC (heat of reaction h_f) were compared with fusion-inhibited vesicles with LPC (heat of reaction h_{fi}). The results are presented in Fig. 2C,D, Fig. 3 and in Table 1. For pH = 6.3 no differences in the reaction heats of liposomes with and without LPC were observed. An example is shown in Fig. 2C where sonicated vesicles (pH 7.4) are injected into buffer at pH 6.3. Vesicles with and without LPC produce identical heats of reaction of $h \approx -50$ µcal which arises exclusively from the pH difference between the two solutions. In separate control experiments LPC solutions (pH 7.4) without other lipids were injected into buffer at low pH. The observed heats of reaction were identical with buffer-into-buffer titrations with the same pH difference.

If the pH in the calorimeter cell falls below pH 6.3 the fusion reaction is initiated and h_f differs from h_{fi}. Fig. 3A then summarizes the pH dependence of the reaction enthalpy ΔH of the fusion process. Note the very steep transition with a midpoint at pH 6.0. ΔH reaches its maximum value at pH $\sim$5.7, i.e. the transition takes place within $\sim$0.6 pH units.

The structural changes accompanying vesicle fusion were further characterized with light scattering and fluorescence resonance energy transfer (RET). The results are summarized in Fig. 3B. The data are normalized according to $\theta = (\theta_{obs} - \theta_{min})/(\theta_{max} - \theta_{min})$ where θ_{min} (θ_{max}) is the minimum (maximum) scattering or fluorescence intensity without (with) fusion. The scattering intensity increases with decreasing pH indicating the formation of larger lipid particles. The change in the light scattering intensity occurs in the same narrow pH interval of ΔpH = 0.6 as detected by isothermal titration calorimetry. For the fluorescence experiment, POPE/OA (7:3 mol/mol) vesicles were prepared which con-

Table 1
pH-induced fusion of lipid vesicles: measurement of the fusion/phase transition enthalpy

Liposomes	T (°C)	pH_{rc}[†]	C_{LPC}[‡] (μM)	n_L·injection^{-1} * (nmol)	Δh_f[+] (μcal)	Δh_{fi}[§] (μcal)	ΔH (cal mol^{-1})
SUV[c]	28	6.7	–	49.4	−32.6	−33.8	∼0
		6.5	–	49.4	−42.0	−38.0	∼0
		6.3[a]	–	49.4[a]	−50.1[a]	−50.3[a]	∼0
		6.0	–	49.4	−38.5	−52.5	280 ± 30
		5.7[a]	–	49.4[a]	−32.7[a]	−57.0[a]	492 ± 50
		5.0	–	49.4	−85.5	−111.5	530 ± 53
		4.0	–	49.4	−80.5	−103.0	460 ± 46
		3.0	–	49.4	−70.0	−101.0	627 ± 63
SUV	50	5.7	–	41.0	−64.0	−78.0	340 ± 60
LUV (50 nm)	28	5.0	–	35.0	−137.2	−155.0	510 ± 80
LUV (400 nm)	28	5.0	–	35.0	−126.0	−146.0	570 ± 90
LUV (100 nm)	28	5.7[b]	50[b]	90.0[b]	−133.0[b]	−75.0[b]	640 ± 70
		5.7[b]	10[b]	90.0[b]	−166.0[b]	−104.0[b]	690 ± 70
SUV	28	5.7	30	50.0	−67.0	−37.0	600 ± 60
		5.7	30	105.0	−110.0	−60.0	480 ± 50

[†]pH in the reaction cell.
[‡]Total LPC concentration in reaction cell.
*Amount of lipid per injection.
[+]Reaction enthalpy in the absence of LPC (or exhaustion).
[§]Reaction enthalpy in presence of LPC.
[a]Presented in Fig. 2.
[b]Presented in Fig. 4.
[c]Presented in Fig. 3.

tained two lipid-bound dyes, NBD-PE and rhodamine-PE, at such concentrations that the NBD fluorescence was efficiently quenched by rhodamine (data not shown). These vesicles were then mixed with non-labeled vesicles of the same composition. Next, the mixture was injected into low pH buffer. Due to vesicle fusion the quencher dye was diluted out and the NBD fluorescence increased. The normalized change of the NBD fluorescence as a function of pH is practically superimposable onto the light scattering data (cf. Fig. 3B, solid symbols). Upon addition of LPC the fusion reaction was inhibited. Neither the light scattering intensity nor the NBD-fluorescence were found to increase (Fig. 3B, open symbols).

Titration calorimetry offers a possibility to determine the critical LPC concentration necessary to inhibit fusion. To this purpose, fusogenic vesicles were titrated into low pH buffer containing LPC at varying concentrations. Only if the LPC concentration in the membrane is sufficiently high will the fusion reaction be blocked. Fig. 4A then shows the titration of liposomes (100 nm vesicles) composed of POPE and oleic acid (7:3 mol/mol; pH 7.4) into a solution of 50 μM LPC at pH 5.7. Each step corresponds to the injection of 20 μl lipid suspension ($C_L \sim 4.5$ mM) into the reaction cell. The lower panel (Fig. 4B) then summarizes the heat of reaction for each step. Upon addition of lipid, LPC rapidly partitions into the lipid membrane. During the first few injections the LPC concentration in the membrane is sufficiently high to inhibit the fusion reaction. With increasing injections of lipid, less and less free LPC will be present. If the free LPC concentration falls below a critical limit, newly added bilayer vesicles will start to fuse. Hence phospholipid vesicles are stable only during the initial phase of the titration experiment and will fuse once the LPC concentration in the aqueous phase is too low. This transition is reflected in the change of the reaction enthalpy. Since the enthalpy of the fusion/phase transition was shown to be endothermic (Fig. 2), the heats of reaction must become less exothermic towards the end of the titration. For the experiment shown in Fig. 4 where $n_L \approx 90$ nmol of lipid were added in each injection, this shift is expected to $\Delta h \approx +0.5$ kcal/mol·90

nmol = +45 μcal which is indeed borne out by the experimental results (Fig. 4B).

The number of lipid injections necessary to exhaust the LPC supply depends on the initial LPC concentration in the calorimeter cell. If it is small, the fusion reaction will be inhibited only for a few titration steps. Fig. 4C shows a titration of a 10 μM LPC solution with lipid vesicles, i.e. the LPC concentration is five times smaller than that used in Fig. 4A. Already after the first injection the LPC concentration falls below the critical limit and the vesicles start to fuse. The lipid concentration in both cases is identical as is the shift in the reaction enthalpy in the two titrations. From the inflection point of the titration curves the critical membrane concentration of LPC needed for inhibition can be derived as will be discussed below.

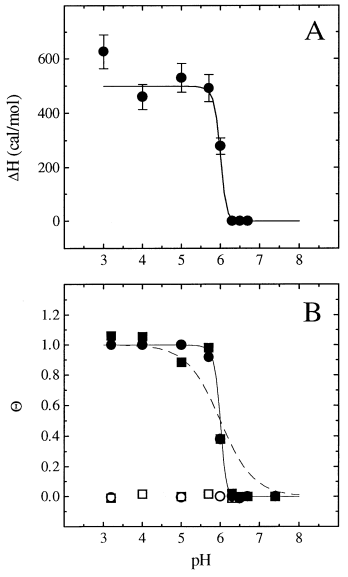

Fig. 3. Lipid vesicle fusion as a function of pH. Vesicles were prepared by sonication in 10 mM Tris, pH 7.4; 100 mM NaCl at a lipid concentration of 4.94 mM. The vesicles were then injected into buffer of the indicated pH. (A) Heats of fusion as measured by titration calorimetry. (B) Spectroscopic measurements using light scattering (■) and fluorescence resonance energy transfer, RET (●). Θ denotes the fraction of fused vesicles calculated as described in the text. Open symbols, LPC treated vesicles, (LPC/lipid = 1:7, mol/mol, LPC added from aqueous stock). T = 28°C. The broken line is the fraction of protonated oleic acid as calculated from protonation equilibrium with an estimated $K_d = 10^{-6}$.

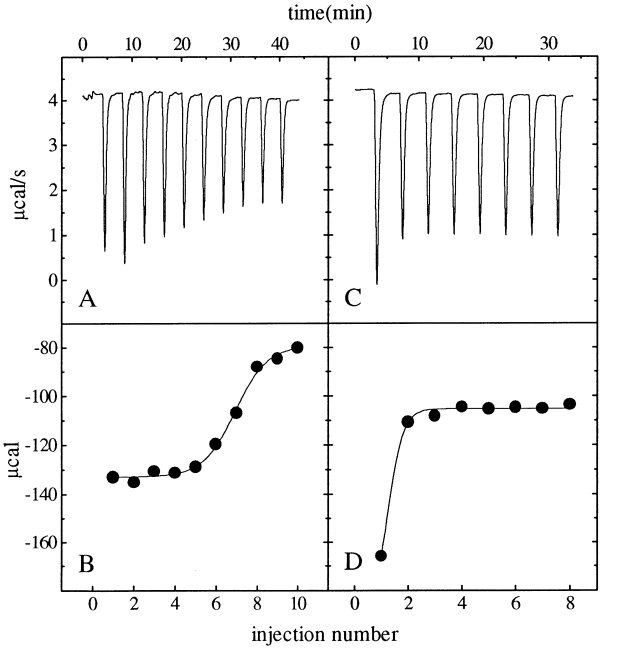

Fig. 4. Titration calorimetry of LPC solutions with vesicles composed of POPE and OA. Lipid suspensions were titrated into the reaction cell containing LPC and buffer at the indicated concentrations and pH values. Upper panels: 20 μl injections of a 4.5 mM lipid vesicles (POPE/OA, 7:3, mol/mol; 100 nm diameter; 10 mM Tris, pH 7.4; 100 mM NaCl) titrated into the reaction cell (V = 1.3353 ml) containing LPC at concentrations of 50 μM (A) or 10 μM (C) in 10 mM MES, pH 5.7; 100 mM NaCl; T = 28°C. The reference cell contained 10 mM MES, pH 5.7; 100 mM NaCl. Downward peaks represent exothermic reactions. Lower panels: heats of reaction obtained by integration of the area underneath the calorimeter tracings (A,C).

3.3. Differential scanning calorimetry

The transition from the lamellar phase to the hexagonal phase of PE containing lipid mixtures can also be induced by increasing the temperature. The enthalpy of transition of pure POPE was determined with differential scanning calorimetry and was found to be $\Delta H \sim 400$ cal/mol [13,14]. We have used the same method to determine the enthalpy of the thermally induced $L_\alpha \rightarrow H_{II}$ transition of the present lipid mixture. The results are summarized in Table 2. Pure POPE membranes showed an $L_\alpha \rightarrow H_{II}$ transition at $T_h = 71 \pm 0.5$°C with a transition enthalpy of $\Delta H \approx 400 \pm 50$ cal/mol, in excellent agreement with previous results [13,14]. Table 2 demonstrates that the addition of oleic acid decreases T_h and increases

Table 2
Temperature-induced lamellar (L_α) to hexagonal (H_{II}) phase transition of POPE in mixture with oleic acid

Oleic acid (mol %)	T_h (°C)	ΔH (cal/mol)
0	70.4 ± 0.5	390 ± 50
	71.8[a]	360[a]
	71.2[b]	420[b]
5	65.0	560
10	63.6	470
15	56.3	580
20	55.0	470
23	58.0	440
26	57.8	600
30	56.0	700

[a]Taken from [13].
[b]Taken from [14].

the transition enthalpy. In the range 0–30% OA the enthalpy of transition varied between 400 and 700 cal/mol (Table 2).

4. Discussion

4.1. Vesicle fusion and the $L_\alpha \rightarrow H_{II}$ phase transition

The physical–chemical properties of lipid mixtures containing phosphatidylethanolamines (PE) have been investigated extensively in the past and a large body of work has been reported on the phase transition [7,23,35,36] and fusion of pure lipid membranes containing PE [8,35–40]. Recently, the proton induced fusion of vesicles composed of PE and oleic acid was investigated [19]. These liposomes were found to be highly sensitive to pH leading to membrane fusion when the pH of the medium was lowered. In the present experiments we have compared the fusion process with the pH-induced $L_\alpha \rightarrow H_{II}$ transition of multilamellar lipid dispersions. The two transitions were found to share common features. Firstly, both transitions occur in the same narrow pH interval of pH 6.3 to 5.7. The transition is most probably triggered by the protonation of oleic acid eliminating the electrostatic repulsion between adjacent bilayers and vesicles. However, the transition range of $\Delta pH \approx 0.6$ is distinctly narrower than that of a normal acid-base titration (cf. dashed line in Fig. 3B). Secondly, the enthalpy of the fusion reaction of $\Delta H = 0.5 \pm 0.2$ kcal/mol (30°C; cf. Table 1) is in good agreement with the enthalpy of the $L_\alpha \rightarrow H_{II}$ transition with $\Delta H = 0.7 \pm 0.1$ kcal/mol for PE/OA = 7:3 ($T_h = 56$°C). Thirdly, both transitions can be inhibited upon addition of sufficient amounts of LPC. Finally, light scattering and fluorescence experiments reveal that vesicle fusion is accompanied by an instantaneous formation of large vesicle aggregates with rapid intervesicle lipid exchange.

Fusion of two or more small lipid vesicles into a single large vesicle will generate non-bilayer structures only as short-lived intermediates. The enthalpy consumed in creating the transition state will be returned upon disintegration of the non-bilayer intermediates. The bilayer-to-bilayer fusion process should thus exhibit an overall enthalpy of close to zero unless the relief of vesicle strain is accompanied by an additional ΔH. However, the measurements of the fusion reaction as a function of vesicle size yield the same ΔH (within experimental error) for vesicle diameters between 30 and 400 nm (cf. Table 1).

Taken together, the data thus suggest that the proton-induced fusion of unilamellar lipid vesicles is, in fact, a rapid aggregation process followed immediately by a transformation of the aggregates into an extended H_{II} phase. The fusion process is identical to the $L_\alpha \rightarrow H_{II}$ transition of coarse bilayer dispersions of the same chemical composition.

4.2. Critical LPC concentration inhibiting fusion

From the experiment shown in Fig. 4 it can be concluded that partitioning of LPC into the lipid membrane is faster than vesicle fusion. Fast partitioning of LPC into the lipid bilayer was also found in experiments where fusion of liposomes was inhibited both by either pre-incubating the liposomes with LPC or co-exposure to both LPC and the fusion trigger (N-terminal part of SIV/HIV fusogenic protein) simultaneously [33].

The partitioning of LPC into the lipid membrane is entropy-driven. This was evidenced in the present work by injecting liposomes into solutions of LPC at non-fusogenic pH. The heat of reaction was found to be zero within experimental error (data not shown). Since no heat is developed, the partioning of LPC into the lipid membrane will not interfere with the calorimetric assay of the fusion reaction.

The titration experiments shown in Fig. 4 allow the evaluation of the critical LPC concentration in the POPE/OA vesicle membrane necessary to inhibit fusion. The partitioning of LPC into the lipid membrane can be described quantitatively by a partition equilibrium of the form $X_b = K_p\ C_{D,f}$. The degree of binding after i injections is denoted $X_b^{(i)}$ and is defined as the molar amount of LPC, $n_{D,b}^{(i)}$, bound per mol of total lipid, $n_L^{(i)}$.

$$X_b^{(i)} = \frac{n_{D,b}^{(i)}}{n_L^{(i)}} = K_p C_{D,f}^{(i)} \quad (1)$$

Here K_p is the partition coefficient for LPC partitioning into the membrane, $c_{D,f}^{(i)}$ is the free (equilibrium) concentration of LPC, and i is the number of lipid vesicle injections. Mass conservation of LPC requires $n_D^o = n_{D,b}^{(i)} + n_{D,f}^{(i)}$ leading to

$$X_b^{(i)} = \frac{K_p C_{D,o}^{(i)}}{1 + K_p C_{L,o}^{(i)}} \quad (2)$$

Here $C_{D,o}^{(i)}$ and $C_{L,o}^{(i)}$ are the total LPC and lipid concentrations, respectively, after i injections. The partition coefficient has been determined as $K_p \approx 5 \times 10^5$ M^{-1} [41]. For the experimental conditions of Fig. 4 $K_p C_{L,o} \gg 1$ and Eq. 2 simplifies to

$$X_b^{(i)} \approx C_{D,o}^{(i)} / C_{L,o}^{(i)} \quad (3)$$

Considering first Fig. 4C, the fusion reaction is inhibited just for the first injection. A degree of binding of $X_b^{(1)}$ is calculated using Eq. 3. The degree of LPC binding after the second injection is $X_b^{(2)}$ and is already too low to inhibit fusion. Hence, the critical degree of binding still inhibiting fusion is about $X_b \sim 0.15$. In the experiment shown in Fig. 4A, the LPC concentration is 50 μM. The fusion reaction is inhibited during the first five injections reaching $X_b^{(5)} \approx 0.158$. After the sixth injection, $X_b^{(6)} \approx 0.134$, and fusion is again possible. This was also confirmed by pre-incubation of liposomes with LPC (data not shown). The above evaluation of the degree of binding was based on the total amount of lipid (POPE and OA) in both half-layers of the lipid vesicle. However, the transbilayer movement of lysophosphatidylcholine has been shown to be very slow [42,43]. For the titration experiments shown in Fig. 4 it can thus be expected that LPC partitions into the outer monolayer only. The outer leaflet contains ~60% of the total lipid. The critical LPC/lipid ratio in the outer leaflet lipid then is ~0.24. This value is close to the amount of oleic acid in the membrane (30%) and, within experimental error, suggests a 1:1 stoichiometry of fusion promoter (OA) and fusion inhibitor (LPC). A compensatory effect at equimolar amounts of both molecules has recently also been found in cell–cell fusion experiments [1]. Further support for a 1:1 stoichiometry follows from the hydrolysis of phospholipids by phospholipase A_2 leading to the production of equimolar amounts of LPC and free fatty acid [44]. The individual components act as detergents, but the equimolar mixture of the two retains the bilayer structure [45,46]. In model experiments pure LPC/fatty acid mixtures in a stoichiometric composition of 1:1 were also found form stable bilayer membranes as evidenced by deuterium and phosphorus NMR spectroscopy [47].

5. Concluding remarks

NMR spectroscopy and high sensitivity titration calorimetry provide evidence that the fusion of unilamellar PE/OA vesicles entails the formation of hexagonal lipid structures. The enthalpy of vesicle fusion is found to be identical to the enthalpy of the $L_\alpha \rightarrow H_{II}$ phase transition. The inhibition of the $L_\alpha \rightarrow H_{II}$ transition and of membrane fusion due to the incorporation of LPC into the bilayer membrane requires about stoichiometric LPC/OA ratios.

Acknowledgements

This research was supported by the Swiss National Science Foundation Grant 31.42058.94.

References

[1] L. Chernomordik, Chem. Phys. Lipids 81 (1996) 203–213.
[2] J. Wegener, H.J. Galla, Chem. Phys. Lipids 81 (1996) 229–255.
[3] D.P. Siegel, Biophys. J. 49 (1986) 1171–1183.
[4] D.P. Siegel, Biophys. J. 49 (1986) 1155–1170.

[5] A.J. Verkleij, Biochim. Biophys. Acta 779 (1984) 43–63.
[6] P.R. Cullis, M.J. Hope, C.P.S. Tilcock, Chem. Phys. Lipids 40 (1986) 127–144.
[7] M.W. Tate, S.M. Gruner, Biochemistry 26 (1987) 231–236.
[8] H. Ellens, D.P. Siegel, D. Alford, P.L. Yeagle, L. Boni, L.J. Lis, P.J. Quinn, J. Bentz, Biochemistry 28 (1989) 3692–3703.
[9] D.P. Siegel, J.L. Burns, M.H. Chestnut, Y. Talmon, Biophys. J. 56 (1989) 161–169.
[10] K. Gawrisch, L.L. Holte, Chem. Phys. Lipids 81 (1996) 105–116.
[11] J.M. Seddon, G. Cevc, D. Marsh, Biochemistry 22 (1983) 1280.
[12] R.M. Epand, Biochemistry 24 (1985) 7092–7095.
[13] R.M. Epand, Chem. Phys. Lipids 52 (1990) 227–230.
[14] R.M. Epand, R. Bottega, Biochemistry 26 (1987) 1820–1825.
[15] J.N. Israelachvili, Biochim. Biophys. Acta 469 (1977) 221–225.
[16] J.N. Israelachvili, D.J. Mitchell, Biochim. Biophys. Acta 389 (1975) 13–19.
[17] G.L. Kirk, S.M. Bruner, D.L. Stein, Biochemistry 26 (1984) 3267–3276.
[18] R.P. Rand, V.A. Persigian, Biochim. Biophys. Acta 988 (1989) 351–376.
[19] N. Duzgunes, R.M. Straubinger, P.A. Baldwin, D.S. Friend, D. Papahadjopoulos, Biochemistry 24 (1985) 3091–3098.
[20] M.J. Hope, M.B. Bally, G. Webb, P.R. Cullis, Biochim. Biophys. Acta 812 (1985).
[21] L.D. Mayer, M.J. Hope, P.R. Cullis, Biochim. Biophys. Acta 858 (1986) 161–168.
[22] T. Wiseman, S. Williston, J.F. Brandts, L.N. Lin, Anal. Biochem. 179 (1989) 131–137.
[23] J. Gagne, L. Stamatatos, T. Diacovo, S.W. Hui, P.L. Yeagle, J.R. Silvius, Biochemistry 24 (1985) 4400–4408.
[24] K. Gawrisch, V.A. Parsegian, D.A. Hajduk, M.W. Tate, S.M. Graner, N.L. Fuller, R.P. Rand, Biochemistry 31 (1992) 2856–2864.
[25] W. Niederberger, J. Seelig, J. Am. Chem. Soc. 98 (1976) 3704–3706.
[26] J. Seelig, Biochim. Biophys. Acta 515 (1978) 105–140.
[27] P. Cullis, B. de Kruijff, Biochim. Biophys. Acta 559 (1979) 399–420.
[28] T.D. Madden, P.R. Cullis, Biochim. Biophys. Acta 684 (1982) 149–153.
[29] P.L. Yeagle, T.S. Fraser, J.E. Young, D. Flanagan, Biochemistry 33 (1994) 1820–1827.
[30] L.V. Chernomordik, S.S. Vogel, A. Sokoloff, H.O. Onaran, E.A. Leikina, J. Zimmerberg, FEBS Lett. 318 (1993) 71–76.
[31] J.L. Nieva, F.M. Goni, A. Alonso, Biochemistry 32 (1993) 1054–1058.
[32] L. Chernomordik, M.M. Kozlov, J. Zimmerberg, J. Membr. Biol. 146 (1995) 1–14.
[33] I. Martin, J.M. Ruysschaert, Biochim. Biophys. Acta 1240 (1995) 95–100.
[34] S. Günther-Ausborn, A. Praetor, T. Stegmann, J. Biol. Chem. 270 (1995) 29279–29285.
[35] H. Ellens, J. Bentz, F.C. Szoka, Biochemistry 25 (1986) 4141–4147.
[36] H. Ellens, J. Bentz, F.C. Szoka, Biochemistry 25 (1986) 285–294.
[37] C. Pryor, M. Bridge, V.A. Persegian, Biochemistry 24 (1985) 2203–2209.
[38] H. Ellens, J. Bentz, F.C. Szoka, Biochemistry 23 (1984) 1532–1538.
[39] H. Ellens, J. Bentz, F.C. Szoka, Biochemistry 24 (1985) 3099–3106.
[40] R. Leventis, T. Diacovo, J.R. Silvius, Biochemistry 26 (1987) 3267–3276.
[41] H.U. Weltzien, Biochim. Biophys. Acta 559 (1979) 259–287.
[42] A.M.H.P. Besselaar, H. Van den Bosch, L.L.M. Van Deenen, Biochim. Biophys. Acta 465 (1977) 454–465.
[43] B. De Kruijff, A.M.H.P. Van den Besselaar, L.L.M. van Deenen, Biochem. Biophys. Acta 465 (1977) 433–453.
[44] G.H. de Haas, L.L.M. van Deenen, Biochem. Biophys. Acta 84 (1964) 471–474.
[45] M.K. Jain, C.J.A. van Echteld, F. Ramirez, J. de Gier, G.H. de Haas, L.L.M. van Deenen, Nature 284 (1980) 486–487.
[46] M.K. Jain, G.H. de Haas, Biochem. Biophys. Acta 642 (1981) 203–211.
[47] P.R. Allegrini, G. van Scharrenburg, G.H. van Haas, J. Seelig, Biochem. Biophys. Acta 731 (1983) 448–455.