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Phospholipase A_2 activity towards vesicles of DPPC and DMPC–DSPC containing small amounts of SMPC

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Abstract

Phospholipase A_2 (PLA_2) is an interfacially active enzyme whose hydrolytic activity is known to be enhanced in one-component phospholipid bilayer substrates exhibiting dynamic micro-heterogeneity. In this study the activity of PLA_2 towards large unilamellar vesicles composed of DPPC:SMPC and DMPC:DSPC:SMPC is investigated using fluorescence and HPLC techniques. Phase diagrams of the mixtures are established by differential scanning calorimetry and the PLA_2 activity, monitored by the lag time, is correlated with the phase behavior of the mixtures. In addition, the degree of lipid hydrolysis in the DMPC:DSPC:SMPC lipid mixtures is detected by HPLC. The PLA_2 activity is found to be significantly increased in the temperature range of the coexistence region where the lipid mixtures exhibit lateral gel–fluid phase separation. Furthermore, in the entire temperature range it is demonstrated that PLA_2 preferentially hydrolyzes the short chain DMPC lipid. This discriminative effect becomes less pronounced when the asymmetric lipid SMPC is present in the lipid substrate. Inclusion of SMPC into either DPPC or DMPC:DSPC vesicles prolongs the lag time. The results clearly show that the PLA_2 activity is significantly enhanced by lipid bilayer micro-heterogeneity in both one-component and multi-component lipid bilayer substrates. The PLA_2 activity measurements are discussed in terms of dynamic gel–fluid lipid domain formation due to density fluctuations and static lipid domain formation due to gel–fluid phase separation.

Keywords: Lipid bilayer; Phospholipid; Micro-heterogeneity; Phase separation; Phase coexistence; Phospholipase A_2; Interfaces; Fluorescence; High performance liquid chromatography

1. Introduction

The lipid bilayer, which consists of a variety of lipids, serves as a host for numerous biological processes taking place in association with cell membranes [1,2]. Several studies using well-defined lipid bilayer systems have focused on elucidating the structural and dynamical properties of lipid bilayer membranes and in particular the influence on biological processes such as protein and enzyme activity [3]. It

Abbreviations: DMPC, dimyristoyl-*sn*-phosphatidylcholine; DPPC, dipalmitoyl-*sn*-phosphatidylcholine; DSPC, distearoyl-*sn*-phosphatidylcholine; SMPC, stearoylmyristoyl-*sn*-phosphatidylcholine; DSC, differential scanning calorimetry; HPLC, high performance liquid chromatography; LUV, large unilamellar vesicle; MLV, multilamellar vesicle

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is well known that one-component lipid bilayers display a number of thermotropic phase transitions which might be of importance for the biological functioning of membranes [4,5]. In particular, the chain melting transition which takes the lipid bilayer from an ordered gel to a more dynamic fluid state has been subject to intensive studies [6–9]. Evidence of dynamic lipid domain structures on the nanometer scale in the vicinity of the main phase transition temperature, T_m, has encouraged speculations of the biological relevance of phase changes that is either of thermotropic or compositional origin [10–14]. It has been shown that several membrane bound proteins exhibit activities that are intimately related to the existence of a small-scale lipid structure [15]. In one-component systems, density fluctuations and dynamic gel and fluid lipid domain formation in the proximity of the main phase transition temperature, T_m, can give rise to small-scale structures in the nanometer range [8,10]. In the case of multi-component lipid mixtures, the mixing properties of the different lipids may lead to equilibrium and non-equilibrium phase separation and gel–fluid domain formation on different length and time scales [16–19].

An example of a binary lipid mixture that exhibits small-scale structure based on gel–fluid phase coexistence is that of dimyristoyl-*sn*-phosphatidylcholine:distearoyl-*sn*-phosphatidylcholine (DMPC:DSPC) vesicles [13,18,20–22]. Even though the phase diagram indicates macroscopic phase separation in a wide concentration and temperature range, several studies including visualization by atomic force microscopy clearly show that the bilayer is highly heterogeneous in the coexistence region, both in acyl chain order and composition [17,19,21,23,24]. In multi-component systems, micro-heterogeneity is provided by the coexistence of phases enriched in predominantly one of the lipid components. Important physical quantities that are of relevance for lipid domain formation in lipid mixtures include, e.g., lipid diffusion lifetimes, lipid bilayer curvature [25] as well as the acyl chain difference between the unlike lipids in the mixture [13,19,22]. In the present study the thermodynamic phase behavior of lipid bilayer systems containing an asymmetric lipid is characterized and related to the functional behavior of a lipid membrane active enzyme, phospholipase A_2 (PLA_2).

Stearoylmyristoyl-*sn*-phosphatidylcholine (SMPC) lipids which have two chains of unequal length attached to the glycerol backbone belong to the class of asymmetric phospholipids. These are abundant lipids in biological membranes [26–30]. Their mixing properties with equal length acyl chain phospholipids [31,32] and their special ability to form interdigitated bilayer membranes have been systematically investigated [33–35]. Interestingly, many asymmetric phospholipids are fully miscible with symmetric phospholipids in the entire concentration range [32]. In this paper, it is studied how the phase behavior of the lipid bilayer substrate in binary and ternary mixtures containing SMPC is related to the activity of the lipid bilayer associated PLA_2 enzyme. This is done by the incorporation of small amounts of SMPC into either dipalmitoyl-*sn*-phosphatidylcholine (DPPC) or DMPC:DSPC vesicles. It is expected that SMPC will position itself at the interfaces between the relatively thin fluid disordered and the thicker gel ordered domains of DPPC, or at the interfaces of gel–fluid phase DMPC and DSPC mixtures. This is expected to minimize the hydrophobic mismatch between the acyl chains. It has been predicted that SMPC mix ideally into both the gel and fluid phases of DPPC lipid bilayers [27,32].

PLA_2 is a small water soluble and lipid bilayer active enzyme that catalyzes the hydrolysis of phospholipids into lysolipids and fatty acids [36]. Several studies have clearly shown that PLA_2 is interfacially activated, which means that the activity is enhanced when the substrate is presented in an aggregated form as compared to the activity towards monomeric substrates [36,37]. Furthermore, the hydrolysis activity on vesicular substrates is significantly influenced by the presence of lipid domains and defects in the bilayer. These defects can be in the form of holes in the bilayer as shown by AFM [38–40], or line defects that are occurring when the bilayer undergoes density fluctuations near the main phase transition temperature, T_m [14,41,42]. In fact, most bilayer defects, being either compositional or structural in origin, increase the PLA_2 activity [43,44]. In general the PLA_2 hydrolysis profile towards vesicular substrates is characterized by a period of slow hydrolysis described by a lag time which is highly dependent on the lateral organization of the lipid bilayer substrate [45,46]. A reaction burst at which the hydrolysis rate

increases by several orders of magnitude follows the lag time [45,47].

PLA_2 activity measurements can therefore be used to probe lipid bilayer micro-heterogeneity, and in the present study we shall take advantage of this property by varying the lipid bilayer composition systematically and measuring the PLA_2 lag time at temperatures were the mixed lipid bilayer substrates are known to display gel–fluid phase separation. In the case of DPPC, the lateral heterogeneity is dynamic in origin, while in the case of the DMPC:DSPC mixture the substrate is expected to exhibit phase separation in terms of static lipid bilayer heterogeneity. Particular attention is paid to the influence of small amounts of SMPC on the PLA_2 activity. SMPC is expected to act as an in-plane surfactant as described above. Differential scanning calorimetry (DSC) is applied to determine an apparent phase diagram and the PLA_2 lag time determined by fluorescence spectroscopy is related to the phase diagram. The degree of hydrolysis of the individual lipid components in the lipid bilayer substrates is determined by high performance liquid chromatography (HPLC).

2. Materials and methods

2.1. Materials

The phospholipids DMPC, DPPC, DSPC, and SMPC were purchased from Avanti Polar Lipids (Birmingham, AL, USA) and used without further purification. PLA_2 (*Agkistrodon piscivorus piscivorus*) was a gift from Prof. R.L. Biltonen, University of Virginia.

2.2. Preparation of large unilamellar vesicles (LUVs)

Weighed amounts of phospholipids were mixed in chloroform/methanol 1:1 (v/v). The organic solvent was evaporated under a weak stream of nitrogen and kept under vacuum over night. Multilamellar vesicles (MLVs) were formed by adding a buffer consisting of 150 mM KCl, 1 mM NaN_3, 30 μM $CaCl_2$, 10 μM EDTA, and 10 mM HEPES (pH 8) to the dried lipids. Hydration of the MLV lipid mixtures was performed at 55°C for at least 30 min. LUVs with a diameter of 100 nm were formed by extrusion ten times through two stacked 100 nm size polycarbonate filters [48].

2.3. DSC

DSC was performed using a MicroCal MC-2 (Northampton, MA, USA) on samples of 10 mM MLV or 10 mM LUV suspensions at a scan rate of 10°C/h. An appropriate baseline was extracted from the resulting thermograms. The thermograms were not corrected for the fast response time of the calorimeter.

2.4. PLA_2 lag time measurements

The PLA_2 hydrolysis of the LUV membranes was studied using a SLM DMX-1100 spectrofluorometer. Upon excitation at 285 nm the intrinsic emission at 340 nm from tryptophan residues in PLA_2 was monitored [47]. All measurements were carried out using a total lipid concentration of 150 μM and a PLA_2 concentration of 150 nM. The lag time was determined as the time elapsed between PLA_2 addition and a sudden increase in the fluorescence, which marks the onset of the reaction burst (see Fig. 3) [47].

2.5. HPLC quantification

HPLC quantification of the hydrolysis reaction was made using a Waters Millennium 2010 system (Milford, MA, USA) equipped with a Waters 510 pump, a Waters 717 Plus autosampler, and a PL-EMD 950 evaporative light scattering mass detector from Polymer Laboratories (Cheshire, UK) using a 5 μm Phenomenex (Torrance, CA, USA) diol spherical column and a mixture of chloroform/methanol/water (73:23:3, v/v) as isocratic mobile phase. 100 μl lipid suspension samples were retrieved directly from the reaction chamber in the fluorometer several times during the PLA_2 lipid hydrolysis time course. The extracted samples were rapidly mixed in chloroform/methanol/acetic acid/water (2:4:1:1) in order to quench the PLA_2 lipid hydrolysis effectively. After quenching the PLA_2 activity it was confirmed that the composition of the sample did not change by storage. Salts were extracted from the sample by shaking thoroughly with 1 ml water. From the organic phase 20 μl was analyzed by HPLC. The de-

gree of hydrolysis was measured by the reduction in the substrate fraction. Dose/response calibration curves were linear in the used concentration range.

3. Results

The heat capacity, C_p, as a function of temperature for LUVs composed of DPPC and SMPC lipids in a broad composition range is shown in Fig. 1. The heat capacity curves obtained by DSC are obtained at a scan rate of 10°C/h. The five heat capacity curves all exhibit a pronounced maximum just above 40°C where pure DPPC vesicles are known to undergo a highly cooperative phase transition from a gel to a fluid state [6,9]. In the low concentration regime of SMPC contents (up to 5 mol%) a narrow peak can be seen on the high temperature side of the heat capacity peak. This peak is probably due to trace impurities of multilamellar structures in the lipid suspension [5]. As the concentration of SMPC increases, the heat capacity maximum is shifted towards lower temperatures, and the peak is broadened considerably. For small amounts of SMPC the heat capacity curves for mixed LUVs composed of DPPC and SMPC lipids show a weak sign of a pretransition. The effect of SMPC on the thermotropic behavior of LUVs primarily consisting of DPPC can furthermore be seen in the insert of Fig. 1. In this figure the onset and termination points of the transition peaks are plotted as a function of the concentration of SMPC in order to establish a partial phase diagram of the DPPC:SMPC mixture. It is readily seen from the insert of Fig. 1 that with increasing content of SMPC the phase transition temperature decreases

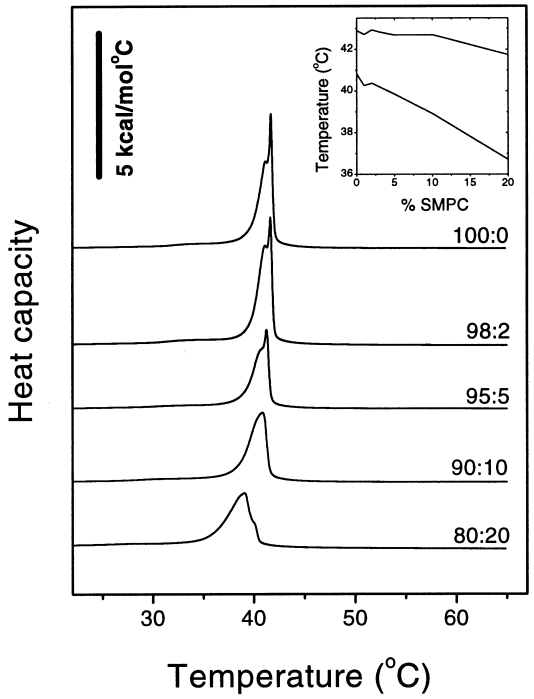

Fig. 1. Heat capacity curves obtained by DSC at a scan rate of 10°C/h of large unilamellar vesicles composed of DPPC and SMPC. The second up-scan of 10 mM lipid vesicles is shown. (Insert) Partial phase diagram for the DPPC:SMPC mixture as determined from the heat capacity curves. The solidus and liquidus lines are obtained by the onset and termination points of the peaks in the DSC thermograms.

Table 1
The transition enthalpy, ΔH, the transition temperature, T_m, and the width of the transition, $T_{1/2}$, of multilamellar vesicles composed of DPPC and SMPC lipids

Composition (mol:mol)	ΔH (kcal/mol)	T_m (°C)	$T_{1/2}$ (°C)
100:0	10.6	41.6	0.09
99:1	9.3	41.7	0.16
98:2	7.9	41.5	0.27
97:3	8.7	41.4	0.27
96:4	8.3	41.3	0.41
95:5	9.3	41.2	0.52
90:10	9.0	40.4	0.95
80:20	9.0	39.7	1.17
70:30	7.2	38.4	1.25
50:50	8.2	36.0	1.44
0:100	6.9	32.2	1.06

The data were obtained by DSC at a scan rate of 10°C/h.

Table 2
The transition enthalpy, ΔH, the transition temperature, T_m, and the width of the transition, $T_{1/2}$, of large unilamellar vesicles composed of DPPC and SMPC lipids

Composition (mol:mol)	ΔH (kcal/mol)	T_m (°C)	$T_{1/2}$ (°C)
100:0	8.0	41.9	1.04
99:1	8.1	41.5	1.23
98:2	8.6	41.6	1.28
97:3	7.1	41.5	1.32
95:5	6.1	41.3	1.42
90:10	7.4	40.8	1.89
80:20	7.6	39.2	2.52

The data were obtained by DSC at a scan rate of 10°C/h.

and the phase transition region is broadened. The values of the enthalpy of the main phase transition determined for the different compositions of MLVs and LUVs are shown in Table 1 and Table 2, respectively. It is seen that the transition temperature decreases monotonically with the mole fraction of SMPC, and that the phase transition region is narrow. Thus, the data in Tables 1 and 2 imply that SMPC mixes ideally into DPPC vesicles, in accordance with earlier work by Bultmann and coworkers [32]. Lipids with a larger chain length difference are predicted to form interdigitated bilayers both by themselves and in lipid bilayer mixtures [27,32].

Fig. 2 shows the heat capacity, C_p, as a function of temperature for mixed unilamellar vesicles for three different compositions of DSPC:DMPC:SMPC. All of the mixtures are characterized by a broad gel–fluid coexistence region reflected by two broad peaks in the C_p curves. Interestingly, the inclusion of SMPC into the equimolar DSPC:DMPC mixed bilayer vesicles decreases the width of the transition peak slightly. It is noticeable that a broad gel–fluid phase coexistence region is still present when a third component, SMPC, is added to the DMPC:DSPC vesicle system. The minor shoulders of the heat capacity traces that consistently were found between the onset and termination points of the transition peaks require further investigation, for example by spectroscopic techniques, in order to determine the structural significance of the peaks in the phase coexistence region. This is outside the scope of the present study which primarily focuses on the relationship between the thermodynamic properties in terms of phase coexistence in the phase transition region and the related PLA_2 activity. The transition enthalpies, ΔH, detected by DSC for the MLVs or LUVs of different DSPC:DMPC:SMPC lipid compositions are listed in Table 3.

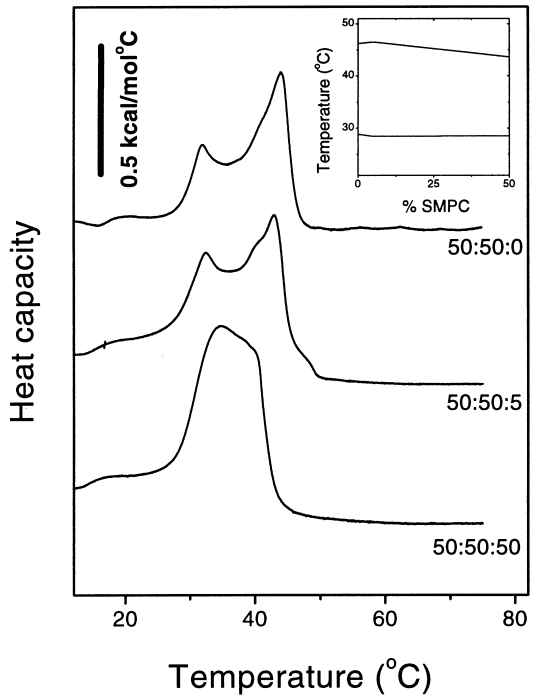

Fig. 2. Heat capacity curves obtained by DSC at a scan rate of 10°C/h of large unilamellar vesicles composed of DMPC, DSPC, and SMPC. The second up-scan of 10 mM lipid vesicles is shown. (Insert) Partial phase diagram for the DMPC:DSPC:SMPC mixture as determined from the heat capacity curves. The definition of the SMPC lipid concentration is [SMPC]/([DMPC]+[DSPC]), where [DMPC] = [DSPC]. The solidus and liquidus lines are obtained by the onset and termination points of the peaks in the DSC thermograms.

Table 3
The transition enthalpy, ΔH, of MLVs and LUVs composed of DMPC, DSPC, and SMPC lipids obtained by DSC at a scan rate of 10°C/h

Composition (mol:mol)	MLV ΔH (kcal/mol)	LUV ΔH (kcal/mol)
50:50:0	9.1	5.6
50:50:5	8.8	5.8
50:50:50	7.7	6.3

In order to relate the thermodynamic measurements by DSC and the established phase diagrams of the mixed lipid bilayers to PLA_2 activity, the duration of the lag phase has been determined as a function of temperature using intrinsic PLA_2 tryptophan fluorescence and HPLC quantification. A representative experiment obtained at 40°C using DMPC:DSPC 50:50 as a lipid substrate is shown in Fig. 3. The apparent phase diagram shown in the insert of Fig. 2 indicates that this lipid substrate exhibits gel–fluid phase separation. The enzyme was added to the reaction chamber ($t = 1200$ s) and the fluorescence emission from the tryptophan residues in PLA_2 causes a fluorescence increase (Fig. 3A). After a certain period, $t = 1700$ s, a sudden increase in the tryptophan fluorescence marks the termination of the lag phase and the onset of the reaction burst [12]. This experimental procedure has been repeated

for each temperature and lipid composition. Temperature equilibration is insured by keeping the lipid suspension at the required temperature at least 30 min prior to adding the PLA_2. Samples for HPLC analysis were retrieved directly from the reaction chamber immediately before PLA_2 addition and several times during the experiment. From the HPLC chromatograms, the area of each lipid fraction peak has been determined and the degree of hydrolysis is calculated from the area difference between the peaks before addition of PLA_2 and at different times after the addition. In Fig. 3B is shown the time dependent degree of hydrolysis in the cases of DSPC and DMPC. It is clearly seen how a rapid increase in the degree of hydrolysis of both lipids coincides in time with the fluorescence increase seen in Fig. 3A. Furthermore, it is evident that DMPC is hydrolyzed to a higher degree than DSPC during the entire time course. This interesting and selective PLA_2 lipid hydrolysis will be discussed in detail later.

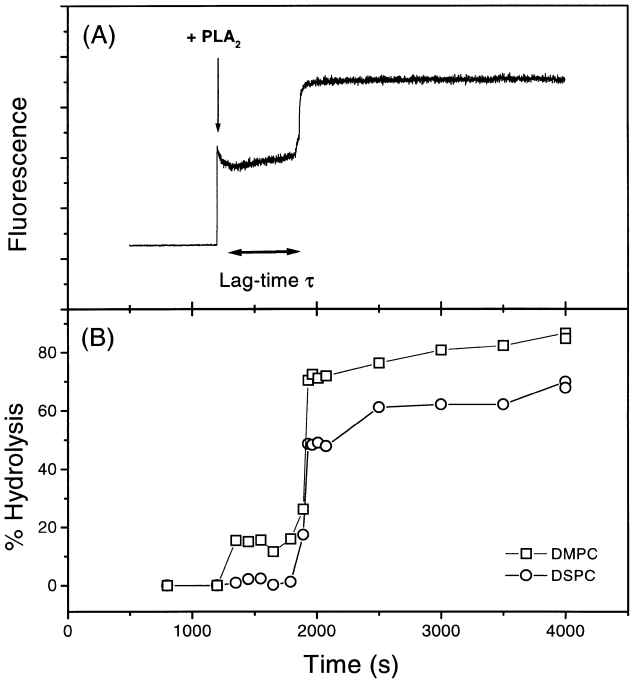

Fig. 3. The characteristic hydrolysis reaction time course at 40°C for PLA_2 acting on DMPC:DSPC mixed large unilamellar vesicles. (A) The PLA_2 hydrolysis reaction is monitored by intrinsic tryptophan fluorescence emitted at 340 nm upon excitation at 285 nm. After adding PLA_2 at time 1200 s to the lipid vesicle suspension, a characteristic lag time, $\tau = 500$ s, follows before a sudden increase in fluorescence signals the burst characterized by rapid lipid hydrolysis by PLA_2. (B) The time dependent degree of hydrolysis is quantified by HPLC analysis of samples retrieved directly from the reaction chamber.

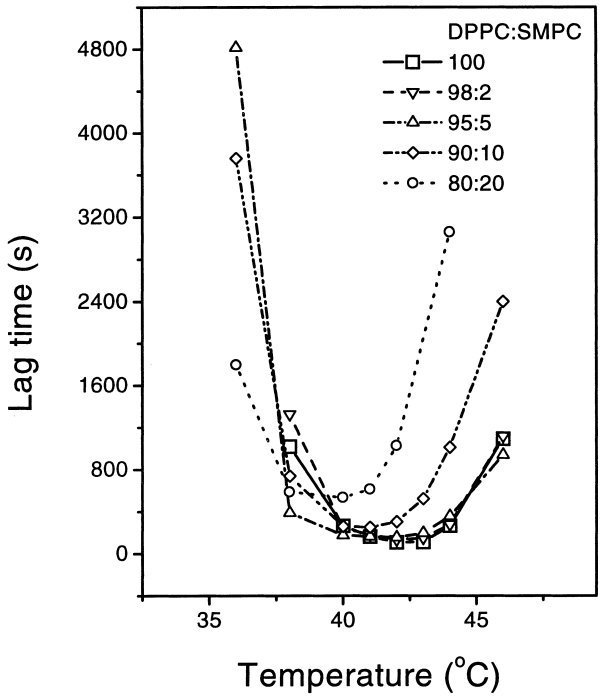

Fig. 4. Lag time, τ, as a function of temperature for the PLA_2 catalyzed hydrolysis of DPPC:SMPC mixed large unilamellar vesicles (100:0, 98:2, 95:5, 90:10, and 80:20). The concentration of PLA_2 is 150 nM and the total lipid concentration of the vesicles is 150 μM.

Fig. 4 shows the resulting lag times as a function of temperature for five different compositions of DPPC and SMPC. Each of the lag time curves exhibits a pronounced minimum. This minimum is found at lower temperatures as the concentration of SMPC is increased. Furthermore, the lag time generally increases with SMPC content. It was confirmed by HPLC (data not shown) that both DPPC and SMPC act as substrates for the PLA_2 catalyzed hydrolysis. This shows that the overall concentration of lipids which can be hydrolyzed does not change with the SMPC content. Hence, the general increase in the lag time that is observed over a broad temperature range is closely related to the lipid bilayer properties and not related to dilution effects.

The lag time data are plotted in Fig. 5 as a function of temperature for three compositions of the mixed DMPC:DSPC:SMPC vesicles. The lag time measurements were performed in a temperature range where the DMPC:DSPC phase diagram indi-

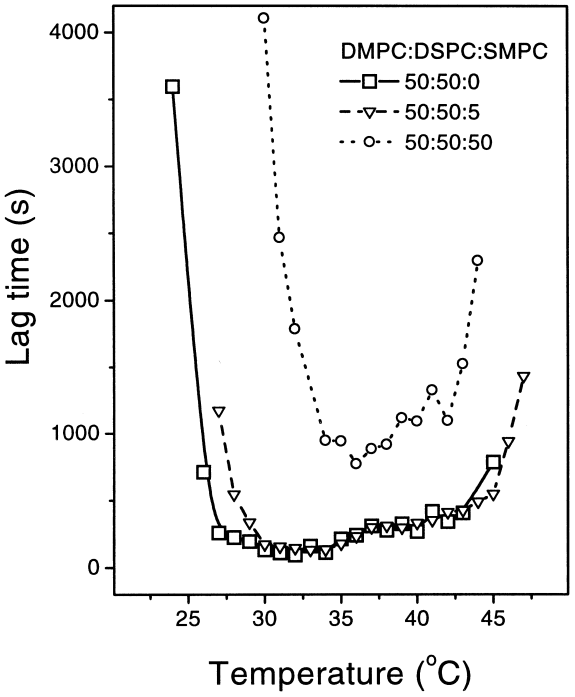

Fig. 5. Lag time, τ, as a function of temperature for the PLA_2 catalyzed hydrolysis of DMPC:DSPC:SMPC mixed large unilamellar vesicles (50:50:0, 50:50:5, and 50:50:50). The concentration of PLA_2 was 150 nM and the total lipid concentration of the vesicles was 150 μM.

cates a two-phase coexistence region. Both in the case of the 50:50:0 and the 50:50:5 mixture, two broad minima can be resolved. From Fig. 5 we find that the PLA_2 activity is highly non-trivial within this two-phase region and presumably is very sensitive to the small-scale structure, i.e. gel and fluid domains, exhibited by the mixed lipid bilayer vesicles. It is furthermore noticeable that the two minima are located at temperatures corresponding to the phase lines of the apparent phase diagram, i.e. the upper liquidus and the lower solidus lines shown in the insert of Fig. 2. In the case of the 50:50:50 mixture, only one minimum is resolved which is narrower than in the case of the 50:50:0 and the 50:50:5 mixtures. In accordance with the lag time results for the DPPC:SMPC mixtures described above, SMPC causes an increase in the lag time in the entire temperature range.

Fig. 6 shows the degree of hydrolysis 1000 s after the detected burst of as a function of temperature for the three lipid mixtures that have been investigated. In the case of the binary DMPC:DSPC mixture that is seen in Fig. 6A, primarily DMPC is hydrolyzed in the entire temperature range. This effect is most pronounced at low temperatures, where the amount of DMPC hydrolyzed is up to 4-fold larger than the amount of hydrolyzed DSPC. The degree of DSPC hydrolysis has a maximum at approx. 45°C, while the maximum DMPC hydrolysis is at 32°C. These temperatures can be directly related to the solidus and liquidus lines of the phase diagram of the mixtures. As SMPC is included in the mixture, the difference in degree of hydrolysis of DMPC and DSPC is diminished. However, for all three mixtures DMPC is still hydrolyzed to the highest extent, although the

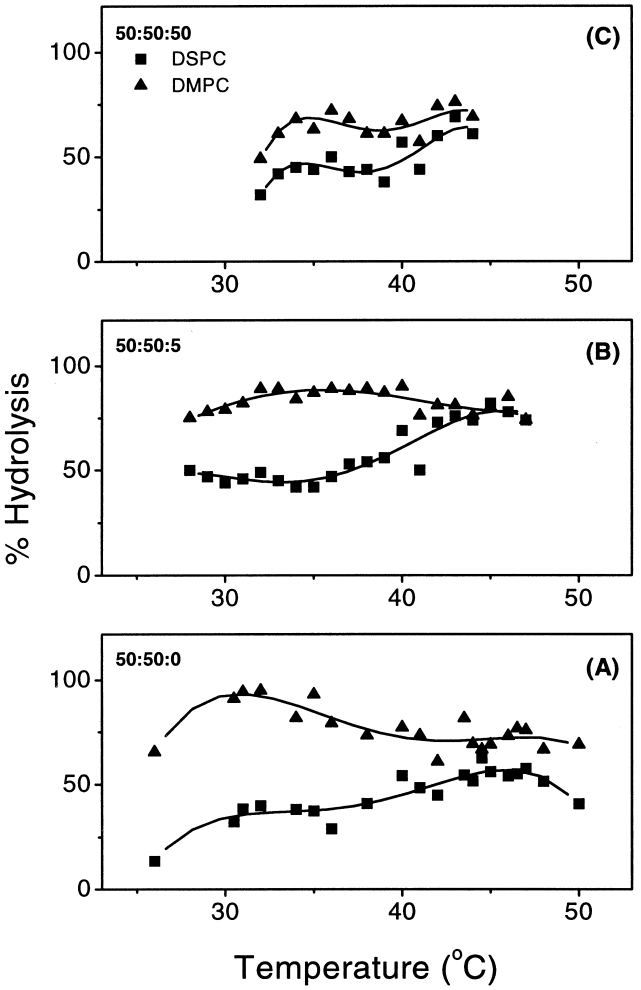

Fig. 6. The degree of PLA_2 catalyzed lipid hydrolysis of DMPC:DSPC:SMPC mixtures as a function of temperature. The degree of hydrolysis of DSPC (□) and DMPC (△) was determined on the basis of HPLC chromatograms (see Fig. 3). (A) 50:50:0; (B) 50:50:5; (C) 50:50:50.

selective hydrolysis of DMPC lipids becomes less pronounced when SMPC is present as revealed by Fig. 6B,C. In the case of the 50:50:50 mixture, DMPC is only hydrolyzed to a slightly higher degree than DSPC.

4. Discussion

The activity of PLA_2 on DPPC:SMPC and DMPC:DSPC:SMPC lipid bilayer mixtures has been determined using HPLC and fluorescence techniques. The degree of lipid hydrolysis and the time required before the fast PLA_2 hydrolysis takes place (the lag time) were compared to the phase behavior of the lipid substrates as determined by DSC. The vesicular systems have been chosen in order to explore the PLA_2 activity under experimental conditions where the lipid substrates exhibit either dynamic micro-heterogeneity due to density fluctuations in the vicinity of the gel–fluid phase transition or static gel–fluid lipid domain formation caused by phase separation in the gel–fluid phase coexistence region. Furthermore, we have investigated the effect of incorporating the asymmetric SMPC lipid that presumably modulates the in-plane interfaces between gel and fluid lipid domains.

The obtained results clearly show that the temperature dependent PLA_2 activity towards one-component DPPC vesicles (see Fig. 4) exhibits a minimum at the phase transition temperature. This lag time minimum has generally been related to the formation of a small-scale structure caused by dynamic gel–fluid domain formation close to the phase transition temperature of the lipid bilayer substrate [14,41,42]. The change in the thermotropic properties of DPPC bilayers upon addition of the asymmetric phospholipid SMPC has been investigated using DSC (see Fig. 1). Clearly, the inclusion of SMPC lipids into DPPC bilayers decreases the main phase transition temperature indicating that SMPC has a slight preference for the fluid bilayer state. This is reflected as a SMPC concentration dependent depression of the transition temperature, T_m, as detected by the peak in the heat capacity. Noteworthy, the transition enthalpy decreases only slightly as a function of the SMPC content and the phase transition temperature range becomes more narrow, suggesting that the transition enthalpy is dominated by the contribution from the chain melting process of both DPPC and SMPC. It should be emphasized that the DSC measurements only provide information about the thermotropic behavior of a vesicle suspension in terms of the second derivative of the free energy, the heat capacity, C_p, from which an apparent phase diagram can be constructed. A true phase diagram can only be obtained on basis of the free energy itself as a function of temperature and composition which in general is unaccessible from experimental measurements. The heat capacity curves of Fig. 1, however, give a clear indication of phase changes in the bilayer vesicles, and furthermore, as earlier predicted [27,32], the results suggest that SMPC lipids mix ideally with DPPC lipids.

Modulating PLA_2 activity by inclusion of a second component into pure DPPC bilayers has been intensively investigated in several studies. For example, the addition of small amounts of cholate [49] into DPPC lipid bilayers results in an increase in the PLA_2 lag time in a broad temperature range. The lag time decreasing effect induced by the second component is generally explained by an increase in the lipid bilayer heterogeneity reflected by a broadening of the gel–fluid phase transition. In the present study, the lag time was determined in a broad range of temperatures for mixtures of DPPC and SMPC (see Fig. 4). A striking relationship was found between the thermotropic data in terms of transition temperature and heat capacity peak width on the one side, and the PLA_2 hydrolysis lag time on the other side (see Figs. 1 and 4). In particular, we find that the minimum in the lag time coincides with the maximum in the heat capacity for all DPPC:SMPC compositions investigated. However, when SMPC is included into the DPPC bilayer, the heat capacity peak broadens and the lag time increases as a function of the concentration of the asymmetric lipid (see Fig. 4). This is an unexpected effect of SMPC on the PLA_2 activity. It could be expected that the inclusion of SMPC decreases the interfacial tension between gel and fluid state domains. This would lead to an enhanced lipid domain formation and PLA_2 mediated hydrolysis activity on substrates exhibiting enhanced micro-heterogeneity [14,41,42]. Although the inclusion of SMPC into DPPC bilayer vesicles causes the main phase transition to broaden and increase

the temperature range over which the bilayer exhibits dynamic gel–fluid micro-heterogeneity, this is not reflected in an increased PLA_2 activity. Attempts have been made to visualize the enhanced PLA_2 activity on lipid structures that exhibit lateral heterogeneity in the vicinity of the gel–fluid phase transition. Fluorescence studies of monolayers are successful for this purpose, because the lipid lateral dynamics are slowed down and a pronounced preference of PLA_2 towards interfacial lateral domain structures has been found [50,51].

The interplay between PLA_2 activity and the formation of interfaces has been investigated further by studying a lipid bilayer mixture composed of DMPC and DSPC exhibiting gel–fluid phase coexistence. The thermotropic behavior of this mixture is shown in Fig. 2 and the corresponding PLA_2 lag times are shown in Fig. 5. For the DMPC:DSPC mixed bilayer, a double minimum in the PLA_2 lag time can be resolved, and the increased PLA_2 activity is confined to the temperature range between the phase lines in the phase diagram of DMPC:DSPC shown in the insert of Fig. 2. Previously, Goormaghtigh and coworkers found a similar increase in PLA_2 activity at the liquidus and solidus lines of the phase diagram of multilamellar vesicles composed of DMPC and DSPC [52].

A systematic quantification of the hydrolysis activity of PLA_2 on the separate lipid species in the mixtures is presented in Fig. 6, showing that a clear selective hydrolysis of the short chain lipid DMPC takes place. As seen in Fig. 3, this discriminative effect is observed both before and immediately after the reaction burst, suggesting that the preferential hydrolysis of DMPC also takes place before the breakdown of the bilayer structure that is known to accompany the reaction burst [47]. Atomic force microscopy studies have shown that PLA_2 preferentially hydrolyzes DMPC in a DMPC:DSPC mixture in the coexistence region [40]. Furthermore, PLA_2 hydrolysis of pure DMPC bilayer substrates generally proceeds with a shorter lag time than in the case of pure DPPC or pure DSPC bilayer substrates [14]. In all three lipid mixtures investigated in this study, DMPC is hydrolyzed to a higher degree than DSPC. The preference for DMPC is most pronounced in the low temperature range. Furthermore, a maximum in the degree of DMPC hydrolysis is seen near the solidus phase line of the phase diagram (see Fig. 6A). At this temperature, domains are formed primarily by fluid state DMPC within a gel state DSPC matrix. As the temperature is raised closer to the liquidus line, the preference for DMPC is markedly decreased, and the degree of specific DSPC hydrolysis has a maximum at 45°C. At this temperature, domains of gel state DSPC are located within a fluid DMPC matrix [23]. The increased degree of hydrolysis of DMPC and DSPC near the solidus and liquidus lines of the phase diagram correlates well with the lag time double minimum shown in Fig. 5. However, although the lag time decreases, and the degree of hydrolysis increases at temperatures near the phase boundaries of the phase diagram, PLA_2 prefers a fluid state substrate for a gel state substrate. How these results relate to the fact that DSPC has longer acyl chains than DMPC, or it is the disordered fluid character of the acyl chain, is not known at present and requires further investigation. To demonstrate whether it is the fluid state or the shorter acyl chain length that governs the degree of hydrolysis, it may prove useful to perform similar experiments on phospholipids with unsaturated acyl chains of a length that is equal to that of the saturated lipid studied.

The intimate relationship between PLA_2 activity and the phase state of the lipid bilayer substrate calls for an interpretation based on the structural properties of the mixed DMPC:DSPC lipid bilayer. The elevated PLA_2 activity on DMPC:DSPC mixtures in the coexistence region certainly implies that a large number of defects exist – possibly as small-scale structures enriched in either gel or fluid phase lipids. This is in agreement with the observations by Leidy and coworkers [21] who found an elevated level of interfaces in the coexistence region probed by fluorescence techniques. Furthermore, studies by Schram and coworkers [23] using fluorescence photobleaching provided experimental evidence of a percolation threshold inside the phase coexistence region of the DMPC:DSPC binary mixture, showing that compartments of fluid and gel domains coexist. Gel and fluid structures of different sizes were later visualized by Bagatolli and Gratton [22] who by means of confocal fluorescence microscopy showed extended domain structures in DMPC:DSPC giant unilamellar vesicles within the gel–fluid coexistence

region. The authors found that the degree of non-ideality in a binary lipid mixture determines the shape and size of the gel–fluid domains [22]. The small-scale structure and in particular the lifetimes and sizes of the lipid domains that can exist in the coexistence region are highly influenced by the interfacial tension arising from the hydrophobic mismatch between the acyl chains. In lipid mixtures governed by a low interfacial tension between gel and fluid domains, the non-equilibrium behavior can lead to the formation of long-lived small-scale structure. In equilibrium, the free energy minimum in a phase coexistence region of a binary lipid mixture gives rise to macroscopic phase separation [24,53] defined by a low amount of interface between a fluid phase and a gel phase. However, it is likely that macroscopic phase separation in a lipid bilayer mixture can only be reached after long equilibration times, much longer than the relevant time scales used in the experimental measurements [19,53]. Certainly, we cannot interpret the results found in Fig. 5 in terms of PLA_2 action on two macroscopically separated phases. On the contrary, the short lag time in the phase coexistence region indicates extensive small-scale structures in the binary mixtures.

The probing of bilayer lateral structure by PLA_2 has been extended to including SMPC into the DMPC:DSPC mixtures. From the results in Fig. 5 it is seen that the PLA_2 activity decreases with the content of SMPC in the DMPC:DSPC mixture similarly to the results obtained with DPPC lipid bilayers shown in Fig. 4. This might suggest similar influences of SMPC on the structure of the one-component DPPC bilayer exhibiting dynamic density fluctuations, and the multi-component DMPC:DSPC bilayer exhibiting static phase separation. It is seen that the hydrolysis activity of PLA_2 is modulated and lowered by inclusion of the asymmetric lipid SMPC into both one-component (DPPC) or multi-component (DMPC:DSPC) lipid bilayer, suggesting a similar effect of SMPC on the one-component and two-component vesicles. Theoretical model calculations for binary lipid mixtures have shown that two-dimensional wetting of the lateral domains leads to a slowing down of the interfacial dynamics and an increase in the lifetime of the domain structure, most likely due to a lowered interfacial tension [13]. The results obtained in this study imply that SMPC is capable of lowering the interfacial tension between gel and fluid domains in both one- and two-component lipid systems, thereby slowing down the interfacial dynamics. It is conceivable that SMPC lipids wetting the gel–fluid interfaces would lead to an increase in PLA_2 hydrolysis lag time in both one- and multi-component systems.

In conclusion, the obtained results show that the duration of the PLA_2 lag time on one-component DPPC vesicles and multi-component DMPC:DSPC vesicles is intimately related to the phase structure of the lipid bilayer substrate. This is manifested in PLA_2 lag time minima within the phase coexistence region and near the phase line of both one- and multi-component lipid bilayer systems. Furthermore, the degree of hydrolysis detected by HPLC reveals an elevated lipid hydrolysis at the phase lines of the DMPC:DSPC:SMPC lipid mixtures. Inclusion of SMPC into the lipid mixtures leads to an increase in the lag time for all the lipid bilayer mixtures investigated, suggesting that SMPC changes the nature of the gel–fluid interfaces and hence the lability of interfacially positioned lipids towards PLA_2 catalyzed hydrolysis.

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