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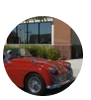

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Phospholipase A_2 activity in dehydrated systems: effect of the physical state of the substrate

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Abstract

In the presence of excess water, enzymatic activity of phospholipase A_2 (PLA_2) depends on the physical state of the lipid substrate. In order to determine if this also holds true in dehydrated systems, the physical parameters of charge, hydration state, and head group spacing of liposome membranes and their effects on PLA_2 lipid hydrolysis were studied. Liposomes of varying composition were freeze-dried in the presence of PLA_2 and partially rehydrated at controlled relative humidities. Accumulation of free fatty acids in the liposomal membranes was used as a measure of PLA_2 activity. We found that PLA_2, which was not activated during lyophilization, was most active during partial rehydration of the liposomes. The hydration state, charge and headgroup spacing of the membrane were all important in determining PLA_2 activity in the dehydrated system.

Keywords: Phospholipase A_2; Liposome; Dehydration

1. Introduction

Nearly all work done on phospholipase activity focuses on enzyme action in the fully hydrated state. There are, however, good reasons to study such enzyme activity in dehydrated membranes. Many organisms that normally survive dehydration accumulate free fatty acids (FFAs) in their membranes during long-term storage [1,2]. These free fatty acids lead to fusion and leakage of cell solutes [2–4]. The de-esterification of fatty acids from phospholipids during storage in the organisms has most often been ascribed to free radical attack on the phosphodiester bond [5–7]. An enzymatic mechanism has been generally ruled out, since it has been thought that there is not sufficient water present in the organisms to support enzymatic activity [1]. Nonetheless, we have provided evidence elsewhere that several phospholipases, including phospholipase A_2 (PLA_2), are active at very low hydration states [8], and we are exploring the possibility that these phospholipases may be active in intact dry membranes. In the present paper we present evidence concerning the physical state of the bilayer under which PLA_2 activity in low water systems is optimal. We have chosen to focus on PLA_2 primarily because it has been so well characterized [9–12].

The data that have been published regarding PLA_2 in systems with limiting hydration have come from non-bilayer experimental systems such as inverted micelles [13–17]. While those findings are consistent with the proposition that PLA_2 may be active in dry organisms, they nevertheless have limited applicability in this context since they do not deal with bilayers. Liposomes are an appropriate model for membranes in the dry state, because they are well-characterized. When dried in the presence of disaccharides, such as trehalose, liposomes retain their barrier properties as well as their bilayer morphology as measured by electron microscopy [18,19], fluorescence spectroscopy [18,20], infrared spectroscopy [21,22], differential scanning calorimetry [22–24], X-ray diffraction [25,26], and NMR [27]. In the dry state, the liposomes exist as intact vesicles, surrounded by a matrix of the sugar [18,19]. Such sugars as trehalose and sucrose are thought to play a similar role in intact dry organisms [22,28–31]. Thus, it has now been possible to study PLA_2 activity on intact, dry liposomes, as we report here.

Because the reaction PLA_2 catalyzes is a hydrolysis, the enzymatic mechanism may differ considerably, de-

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pending on the hydration level of the system, and therefore, the mechanism in low-water systems merits investigation. It is well known that in excess water, the physical state of the lipid substrate is of critical importance in determining the hydrolytic activity of PLA_2. For instance, vesicles of saturated long-chain phospholipid provide a poor substrate for the enzyme above the phase transition temperature of the lipid [32,33]. A partial disruption of the lipid bilayer, however, with bile salts or detergents dramatically increases enzyme activity [34–36]. Increasing detergent concentration still further disperses the lipid aggregates, and will again cause PLA_2 activity to decrease [34,35].

In addition, passing the lipid through its gel to liquid crystalline phase transition, or causing lateral phase separation in the membrane by other means, increases PLA_2 enzymatic activity [37–43]. Finally, physical deformation caused by osmotic shock or a high radius of curvature also augments the hydrolytic activity of PLA_2 [33,39,40,44,45]. Thus, the physical state of the membrane clearly affects PLA_2 activity in the hydrated system.

It is likely that the physical state of the lipid is also important in a system with little water available. Certain early investigations suggest that this is the case. Wells and co-workers [14–16] showed the hydration level to be important in determining PLA_2 activity in inverted micelles. In contrast to lyophilized liposomes, the inverted micelle system was composed of lipids dissolved in diethyl ether with varying amounts of water added. Regardless of the amount of water in the system, however, the suspension of substrate in organic solvent represents an extremely non-biological system. Further, the results regarding PLA_2 in wet ether are not applicable even to other inverted micelle systems. This is illustrated by the finding that PLA_2 cannot be activated in reverse micelles formed in benzene [16]. Thus, data obtained on PLA_2 activity in low water systems using inverted micelles cannot be considered comprehensive for all physical lipid states. The present study probes some aspects of the enzymatic mechanism of PLA_2 on lipid bilayers under limiting hydration conditions. Results indicate that charge, headgroup spacing and hydration state all affect PLA_2 activity.

2. Materials and methods

2.1. Liposome preparation

Dielaidoyl-sn-3-phosphocholine (DEPC), dipalmitoyl-sn-3-phosphocholine (DPPC), dioleoyl-sn-3-phosphocholine (DOPC), 1-palmitoyl-2-oleoyl-sn-3-phosphocholine (POPC), and phosphatidylserine (PS) were purchased from Avanti Polar Lipids (Alabaster, AL), and used without further purification. Lipid was evaporated from chloroform in the appropriate ratios under a stream of nitrogen. The lipid film was placed under vacuum for at least 3 h to remove any residual chloroform. The lipid (10 mg) was rehydrated with 0.5 ml deionized water. This water contained 2 μM calcium as measured by atomic absorption spectroscopy. This solution was warmed in a 60° C water bath for 5 min, then vortexed to produce multilamellar vesicles (MLVs) at 20 mg/ml (25 mM).

Large unilamellar vesicles (LUVs) were produced by extrusion using a hand-held extruder (Avestin, Ottowa, ON, Canada) with 0.1 μm pore filters (Poretics, Livermore, CA). In most cases (exception noted in Results), trehalose (Pfanstiehl Laboratories, Waukegan, IL) was added to a final concentration of 2 g trehalose/g lipid. In experimental samples, phospholipase A_2 from porcine pancreas (Sigma) was included at a concentration of 7 units per mg lipid (12.6 μM).

Small unilamellar vesicles (SUV's) were prepared by sonication of the solution of MLV's in a bath sonicator (model G112SP1T, Laboratory Supplies, Hicksville, NY) until clear.

2.2. Lyophilization

Liposomes were placed in 50-μl aliquots into 1.5 ml Eppendorf tubes (Westbury, NY) with a perforated cap. After rapid freezing in liquid nitrogen, the samples were placed under vacuum on an Edwards Freeze Dryer Super Modulyo (Sussex, UK) for at least 12 h. For the samples held under vacuum for extended periods, sealed vacuum vials were used and the samples were stored in the dark.

2.3. Partial rehydration of liposomes

Relative humidity (RH) was maintained at specified levels by keeping saturated solutions of salts in closed containers. The salts used for specific relative humidities were as follows: potassium acetate for 20%, KCNS for 47%, NaBr for 58%, and sodium acetate for 76%. Liposomes were partially rehydrated by transferring the perforated Eppendorf tubes to racks inside the chambers for specified lengths of time.

2.4. Lipid extraction and analytical thin layer chromatography

Lipid was extracted from the liposomes with chloroform methanol (2:1) and water. Ionic strength of the aqueous phase was increased to approximately 1 M with KCl when necessary. The samples were vortexed vigorously for 30 s, then centrifuged at 15000 × g at 4° C with an Eppendorf Micro Centrifuge 5451 C (Westbury, NY).

The samples were analyzed by analytical thin layer chromatography (TLC) using an Iatroscan TH-10 (Iatron Laboratories, Tokyo, Japan). Extracted lipid in $CHCl_3$ was spotted onto Chromarod S-III Iatroscan rods (Iatron Laboratories) with a 10-μl Hamilton syringe (Hamilton, Reno, NV). The rods were developed in a solvent system of

$CHCl_3$:MeOH:H_2O (65:25:0.5) with 1% acetic acid (v/v) and dried in a 60° C plate-drying oven. The rods were then passed through the hydrogen flame ionization detector and recorded using an E and K Linear chart (Saratoga, CA). Resolution of detection is less than 1 μg. The chart records were quantified by integrating the peaks with a Planix 6 digital planimeter (Tamaya Technics, Tokyo, Japan) and comparing to standard curves of the various lipid species.

2.5. *p*-Bromophenacyl bromide inactivation of PLA_2

According to the method of Volwerk et al. [46], a system of 100 mM sodium cacodylate-HCl and 100 mM NaCl (pH 6.0) was prepared. To a buffer volume of 0.5 ml, PLA_2 was added at 12.6 μM along with *p*-bromophenacyl bromide (Sigma) from a 10-mM stock in ethanol, at a *p*-bromophenacyl bromide to PLA_2 ratio of 50:1. This solution was mixed thoroughly by vortexing and incubated at 30° C for 22 h. The solution containing sodium cacodylate-HCl, NaCl, and *p*-bromophenacyl bromide was considered the 'inactivation buffer'.

2.6. Measuring adsorbed water

Saturated salt solutions of potassium acetate, KCNS, NaBr, and sodium acetate were prepared with tritiated water. Lyophilized liposomes were transferred to the relative humidity chambers containing one of the salt solutions and allowed to incubate at 22° C. Water accumulation by the dry liposomes was measured by counting in Ready Safe Liquid Scintillation Cocktail (Beckman, Palo Alto, CA) on a Beckman LS-100C Liquid Scintillation System with 5-min counts at a 5% standard error.

2.7. Cooling liposomes through the phase transition

Liposomes (50 μl) were placed into 1.5-ml Eppendorf tubes and capped. The tubes were placed in racks inside a Cryomed Regulated Cooling System 910 with a Model 1010 Microcomputer (Custom Biogenics Systems, Shelby Township, MI). The samples were cooled through the phase transition of DEPC (12° C) as follows. From 22° C to 12° C, samples were cooled at 1° C/min. From 12° C to 9° C, samples were cooled at 0.1° C/min. From 9° C to 4° C, samples were cooled at 1° C/min.

2.8. Carboxyfluorescein retention after freeze drying

Carboxyfluorescein (CF) retention by liposomes was used to measure membrane integrity. DEPC liposomes were prepared by extrusion as described above in the presence of 100 mM CF. External CF was removed by column chromatography (Sephadex G 50-80). Trehalose (2 g/g lipid) and PLA_2 (5.4 μM) were added to the liposomes (19 mM DEPC) and 50-μl aliquots were immediately frozen in liquid nitrogen and lyophilized for at least 12 h. The samples were removed from the lyophilizer (at time zero) and exposed to 47% RH in the dark for different periods of time. The liposomes were then rehydrated completely with 10 mM TES at pH 7.5. A small volume of rehydrated liposomes (5 μl) was then transferred to a cuvette containing 3.5 ml TES buffer. Samples were excited at 460 nm, and CF emission was measured at 550 nm on a Perkin-Elmer LS-5 fluorescence spectrophotometer. Intravesicular CF concentrations are self quenching, so the emission at 550 nm was due only to external CF. Percent leakage was calculated after adding 50 μl 1% Triton X-100 to each sample to cause total (100%) leakage. Percent retention was considered to be the difference between 100 and the measured percent leakage.

3. Results

3.1. PLA_2 activity in different states of hydration

When DEPC liposomes were lyophilized in the presence of PLA_2, then completely rehydrated and incubated

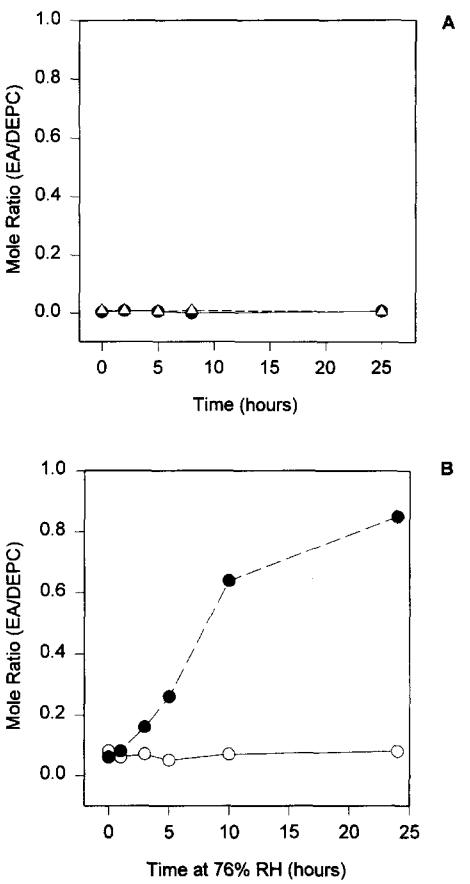

Fig. 1. (A) Elaidic acid production in hydrated DEPC liposomes over time. Samples were allowed to incubate at 22° C in the presence (●) or absence (△) of PLA_2. (B) Elaidic acid production in partially rehydrated lyophilized DEPC liposomes. Samples were allowed to incubate at 22° C and 76% RH in the presence (●) or absence (○) of PLA_2.

at 22° C, the enzyme was inactive. As shown in Fig. 1A, there was no accumulation of FFA in the liposome membrane over a period of 24 h, as measured by analytical TLC. This result was expected, as there was nothing present in the system that would lead to activation of the enzyme (such as passing the liposomes through their phase transition temperature). In a similar set of experiments, fully hydrated DEPC liposomes that had not been freeze-dried were allowed to incubate at 22° C for 24 h. There was no detectible FFA production in either the control samples or those which included PLA_2 (data not shown).

When liposomes freeze-dried in the presence of PLA_2 were allowed to rehydrate partially, however, there was a large accumulation of FFAs in the membrane due to PLA_2 activity. Fig. 1B shows the elaidic acid (EA):DEPC (PC) mol ratio of liposomes allowed to incubate at 22° C and 76% RH over a period of 10 h. It is quite clear that in the presence of PLA_2, there was a dramatic accumulation of FFA in the membrane (at 10 h, EA/PC = 0.64, $n = 4$) compared to the control (at 10 h, EA/PC = 0.07, $n = 4$).

It was also important to know if PLA_2 was activated by the lyophilization procedure itself. The first extraction of freeze-dried liposomes was performed immediately after the samples were removed from the lyophilizer (t_0). Because there was no difference in the EA:DEPC ratios at t_0 between samples freeze-dried in the presence (EA/PC = 0.06, $n = 4$) or absence (EA/PC = 0.08, $n = 4$) of PLA_2 (Fig. 1B), PLA_2 could not have been active during the rapid freezing or lyophilization procedures. The level of hydration, therefore, is one important aspect of the physical state of the system that must be taken into account when considering the mechanism of activation of PLA_2 under limiting hydration states.

In order to determine that the appearance of FFAs in the liposome membranes was due to enzymatic de-esterification by PLA_2, and not some non-specific mechanism such as oxidation or free radical damage, several controls were conducted. As shown in Fig. 2, three conditions were studied: excess calcium (2 mM), negligible calcium (10 mM EGTA), and PLA_2 inactivated by *p*-bromophenacyl bromide.

Results from the excess calcium experiment are shown in Fig. 2A. DEPC liposomes with PLA_2 were formed in the presence of 2 μM Ca (concentration in the deionized water) or 2 mM added $CaCl_2$. After lyophilization, the liposomes were transferred to 76% RH for 24 h. The FFA accumulation in the membrane is shown at 5 h (a: 2 μM Ca; and b: 2 mM Ca) and at 24 h (c: 2 μM Ca; and d: 2 mM Ca). It is clear that including excess calcium does not increase the activity of PLA_2 as measured by FFA accumulation. Thus the 2 μM Ca which is present in the deionized water, when concentrated by lyophilization, provides a calcium concentration sufficient to support PLA_2 activity.

Since water is adsorbed to the sample over time, it would be impossible to maintain a constant calcium concentration throughout the experiment. According to water absorption experiments in this and other studies [8,47], however, the most water that could accumulate per sample in 76% RH over 24 h would be approximately 1 μl. Thus, the minimum possible calcium concentration in these samples would be 100 μM. It is likely, however, that much of this water would be hydrogen-bonded to lipid head-groups, decreasing the free water available and further increasing the calcium ion concentration.

A second control using EGTA to chelate calcium is shown in Fig. 2B. DEPC liposomes with PLA_2 were made

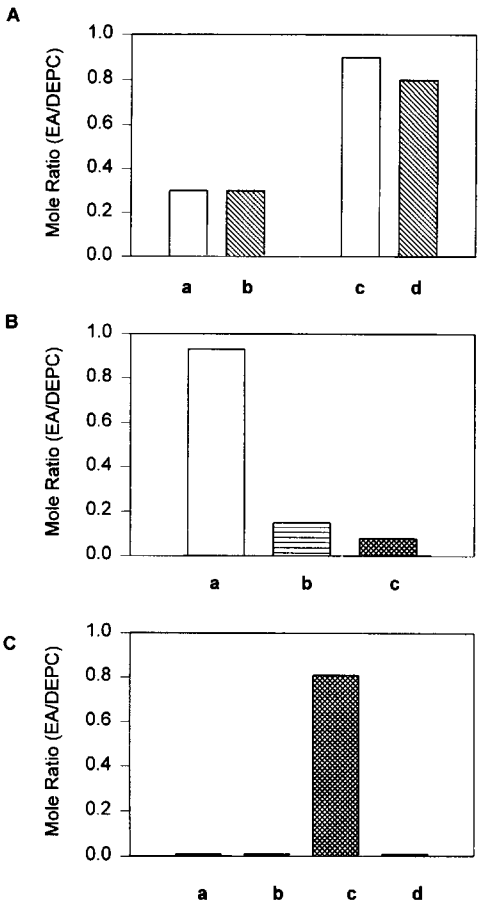

Fig. 2. (A) Elaidic acid production in partially rehydrated lyophilized DEPC liposomes in the presence and absence of excess calcium. Samples were placed in 76% RH for different time periods. Elaidic acid production at 5 h is shown for samples that started with 2 μM Ca (a), or 2 mM Ca (b). Elaidic acid production at 24 h is also shown for samples that started with 2 μM Ca (c), or 2 mM Ca (d). (B) Elaidic acid production in partially rehydrated lyophilized DEPC liposomes in the presence and absence of 10 mM EGTA. Liposomes were placed in 76% RH at 22° C for 24 h. Samples included liposomes prepared with PLA_2 in 2 μM Ca (a), with PLA_2 in 10 mM EGTA (b), and without PLA_2 in the deionized water (c). (C) Elaidic acid production in partially rehydrated lyophilized DEPC liposomes in the presence and absence of *p*-bromophenacyl bromide-inactivated PLA_2. Liposomes were allowed to incubate at 22° C in 76% RH for 24 h. The bars correspond to controls without PLA_2 (a), samples prepared in the inactivation buffer without PLA_2 (b), samples with PLA_2 (c), and samples with the *p*-bromophenacyl bromide-inactivated PLA_2 (d).

in the standard deionized water or 10 mM EGTA (pH 5.7). After lyophilization, they were transferred to 76% RH for 24 h. The elaidic acid (EA) to DEPC mol ratio produced by PLA_2 is shown for the reactions in the absence (a) and presence (b) of EGTA. It can be seen that in the presence of EGTA, the production of FFAs in the membrane is similar to the level often seen in control samples with no PLA_2 included (c).

Third, as shown in Fig. 2C, *p*-bromophenacyl bromide was used to inactivate PLA_2. SUVs were prepared under several conditions noted below and lyophilized, at which point they were placed into 76% RH for 24 h. The measured FFA accumulation is shown for the control samples with no PLA_2 (a), samples prepared with the inactivation buffer but without PLA_2 (b), samples with unmodified PLA_2 (c), and samples with *p*-bromophenacyl bromide-inactivated PLA_2 (d). Since *p*-bromophenacyl bromide is a specific inhibitor of PLA_2, and since the FFA production seen in samples with inactivated PLA_2 was no greater than that seen in control samples, it was concluded that the appearance of FFAs in the liposomal membranes was due to the specific activity of PLA_2, rather than some non-specific mechanism.

3.2. Effects of charge and hydration of membrane

In order to understand better the mechanism of hydrolysis mediated by PLA_2 at low water contents, the physical state of the membrane was varied in two key aspects concomitantly, charge and hydration. Liposomes were prepared in four different DEPC:PS ratios: pure PC, 9:1, 7:3, and 5:5. The liposomes were freeze-dried in the presence or absence of PLA_2, then transferred for 5 h to chambers with the RH held constant at 20%, 47%, 58%, or 76%.

As seen in Fig. 3A, in samples freeze-dried in the absence of PLA_2, no significant increase in the FFA level of the membrane was seen at 5 h at any relative humidity. This result also indicates that non-enzymatic de-esterification of fatty acyl chains is of minimal importance under these conditions.

In the presence of PLA_2, however, there was a large accumulation of FFA in the membrane over 5 h for all PC:PS ratios (Fig. 3B). Both charge and hydration of the membrane affect enzymatic activity, as evidenced by the increase in elaidic acid:phospholipid (EA/PL) ratio in correlation to both relative humidity and the PS concentration in the liposomes. This was expected, as negative charge has been seen before to increase PLA_2 activity [48–50].

Since water availability was limited, however, it was necessary to determine if the effect of charge was simply due to increasing the number of water molecules adsorbed to the membrane. The water associated with the liposome membranes was quantified using tritiated water and scintillation counting. As shown in Table 1, the amount of water associated with the liposomes at 5 h increased with incubation at increasing RHs, as was expected. However, there was no correlation between the PC:PS ratio and the amount of water adsorbed to the liposomal membranes at any

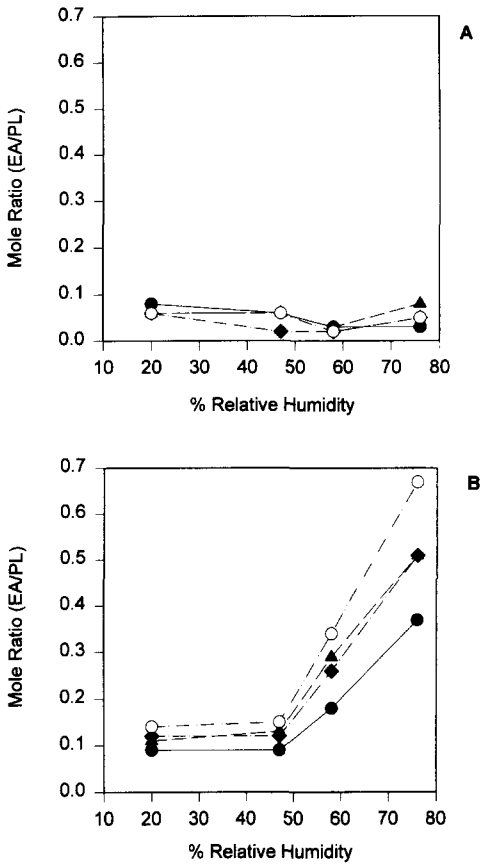

Fig. 3. (A) Elaidic acid production in partially rehydrated lyophilized liposomes of varying DEPC:PS ratios in the absence of PLA_2. Samples of pure PC (●), PC 9:1 PS (▲), PC 7:3 PS (◆), or PC 5:5 PS (○) were allowed to incubate at 22° C for 5 h at 20%, 47%, 58%, or 76% relative humidity. (B) Elaidic acid production in partially rehydrated lyophilized liposomes of varying DEPC:PS ratios in the presence of PLA_2. Samples of pure PC (●), PC 9:1 PS (▲), PC 7:3 PS (◆), or PC 5:5 PS (○) were allowed to incubate at 22° C for 5 h at 20%, 47%, 58%, or 76% relative humidity.

Table 1
Accumulation of water by lyophilized liposomes

DEPC:PS	Relative humidity			
	20%	47%	58%	76%
pure PC (−)	0.55	0.52	0.60	0.78
pure PC (+)	0.57	0.73	0.88	1.10
9:1 (−)	0.51	0.58	0.61	0.81
9:1 (+)	0.56	0.76	0.87	1.22
7:3 (−)	0.37	0.51	0.64	0.71
7:3 (+)	0.37	0.68	0.83	0.87
5:5 (−)	0.31	0.59	0.71	0.77
5:5 (+)	0.45	0.77	0.95	1.02

Liposomes composed of different ratios of DEPC:PS (pure PC, 9:1, 7:3, 5:5) were lyophilized and partially rehydrated by incubating at various relative humidities for 5 h. Water adsorbed to the liposomes was measured with ^3H_2O and is listed as the volume (μl) associated per sample (1 mg lipid) in the presence (+) or absence (−) of PLA_2.

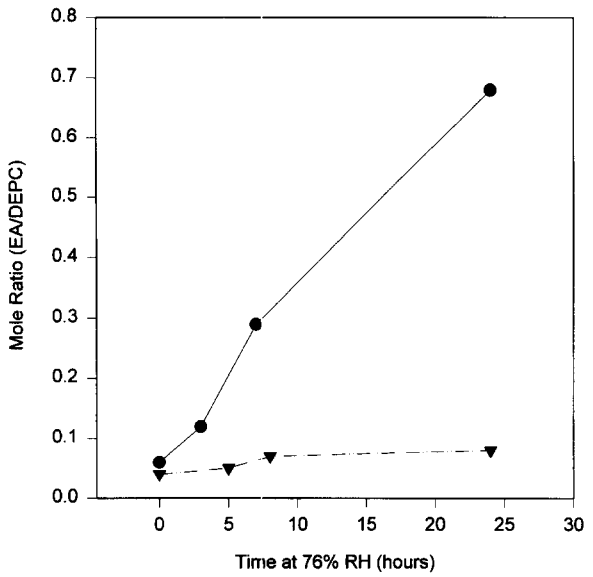

Fig. 4. Elaidic acid production in DEPC liposomes freeze-dried in the presence of PLA_2. Samples were allowed to incubate at 22° C for 5 h at 76% RH. PLA_2 was inactive when lyophilized with liposomes in the absence of trehalose, a procedure leading to the formation of MLVs (▼). There was considerable FFA production when PLA_2 was freeze-dried alone, then rehydrated and lyophilized with liposomes in the presence of trehalose (●). This result indicates that lyophilization in the absence of trehalose does not itself inactivate the PLA_2.

given RH. Hydration and charge, therefore, represent two separate physical parameters in terms of PLA_2 activation in low water systems.

3.3. Large unilamellar vesicles compared with multilamellar vesicles

Another result that indicates the physical state of the membrane may be important in PLA_2 activity is the following. When DEPC liposomes with PLA_2 were freeze-dried in the absence of trehalose, FFA production over a 24 h period at 76% RH was minimal (at 24 h, EA/PC = 0.08, $n = 4$; see Fig. 4). We tested two alternative hypotheses that could explain this result:

(1) Effects of lyophilization on stability of PLA_2

Lyophilizing PLA_2 in the absence of trehalose does not itself inactivate the enzyme. PLA_2 was freeze-dried without trehalose, then rehydrated and added to DEPC liposomes with trehalose. When those liposomes were lyophilized and partially rehydrated at 76% RH, there was a sharp increase in FFA production over time, producing an EA:PC ratio of 0.68 at 24 h ($n = 2$) (Fig. 4).

(2) Activity of PLA_2 on MLVs

In the absence of trehalose, LUVs fuse during freezing or lyophilization, producing MLVs [51]. PLA_2 may have been excluded from or inhibited in the interlamellar spaces of the MLVs, leaving only the external leaflet as substrate for the enzyme, and thus limiting the PLA_2 activity. This effect was also apparent in hydrated DEPC liposomes (Fig. 5). PLA_2 was added externally to LUVs or MLVs. Alternatively, MLVs were also prepared by hydrating dry lipids in water containing PLA_2, providing maximal opportunity for trapping the enzyme between lamellae. Regardless of how the PLA_2 was introduced to the MLVs, essentially identical results were obtained. When the liposomes were cooled through their phase transition over 45 min, there was a dramatically higher FFA production in LUVs (EA/PC = 0.12, $n = 4$) than in either MLV sample (for both, EA/PC = 0.01, $n = 4$). This is likely due to the fact that much more substrate was accessible to PLA_2 in the LUVs. In contrast, in the MLV samples, much of the membrane was sequestered as internal lamellae. Even when enzyme was included during the formation of the MLVs, it was either excluded from the interlamellar spaces, or its activity was inhibited if it was trapped between the lamellae. Fig. 5 also shows that in hydrated samples, the presence or absence of trehalose has little effect on enzyme activity.

3.4. Headgroup spacing affects PLA_2 activity

Further evidence that steric access may be important in determining PLA_2 activity is provided by examining lipids with different headgroup spacing. Since it is known that increasing the level of unsaturation of the fatty acyl chains

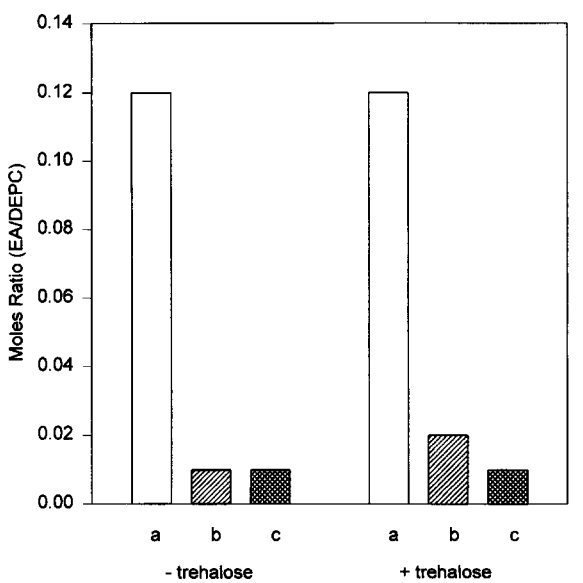

Fig. 5. Elaidic acid production in hydrated DEPC liposomes passed through the gel to liquid crystalline phase transition in the presence of PLA_2. A much larger accumulation of elaidic acid in the liposomal membrane was seen in LUVs (a) than in MLVs to which PLA_2 had been added externally (b) or during preparation (c). The presence or absence of trehalose did not affect PLA_2 activity.

Table 2
Liposomes composed of lipids with increasing per molecule surface area were lyophilized and partially rehydrated at 76% RH for 5 h in the presence or absence of PLA_2

Lipid	−PLA2 (*n*)	+PLA2 (*n*)
DPPC	ND (4)	0.09 (6)
POPC	0.01 (4)	0.14 (4)
DOPC	0.02 (4)	0.39 (4)

Values listed are the mean FFA:PL ratios in extracted membranes as determined by analytical thin layer chromatography with the number of trials included in parentheses. ND indicates values too low to be detected by this method.

increases head group spacing in a bilayer (for review, see Ref. [52]), liposomes composed of DPPC, POPC, or DOPC were freeze-dried and partially rehydrated for 5 h at 76% RH. As shown in Table 2, the free fatty acid to phospholipid ratio (FFA:PL) at 5 h increased in correlation with increasing surface area per phospholipid. Values ranged from 0.09 ($n = 6$) in DPPC to 0.39 ($n = 4$) in DOPC. This finding suggests that increased head group spacing may provide less obstructed access to the *sn-2* bond of the phospholipid for PLA_2, resulting in a higher level of enzyme activity.

3.5. Storage under vacuum is non-static

In order to determine if liposomes freeze-dried in the presence of PLA_2 and held under vacuum represented a physically static system, long-term storage studies were done. Liposomes (DEPC 9:1 PS) were lyophilized in the presence or absence of PLA_2, sealed under vacuum and stored in the dark for up to 45 days. The liposomes were either transferred to a 76% RH chamber for 5 h, extracted and measured with analytical TLC, or extracted and measured immediately.

The ratio of the experimental elaidic acid/phospholipid mol ratio (+PLA_2) to control elaidic acid/phospholipid mol ratio (−PLA_2) for both time points are shown in Fig. 6. It is clear that PLA_2 was never active during storage, because the experimental/control ratio (mole $ratio_{ex}$/mol $ratio_c$) was always near 1.0 for the t_0 extractions. The experimental/control ratio of the 5 h measurements decreased with increasing storage time (at 1 day, mol $ratio_{ex}$/mol $ratio_c = 7.0$, $n = 7$; at 45 days, mol $ratio_{ex}$/mol $ratio_c = 3.3$, $n = 3$). This decrease in experimental: control ratio seen over time was not due to an increase in control values. The EA/PL ratio of control samples, extracted after 5 h at 76% RH, were extremely constant (at 1 day, EA/PL = 0.08, $n = 7$; at 45 days, EA/PL = 0.08, $n = 3$).

This result indicates that PLA_2 may lose some of its catalytic efficacy during storage under vacuum. The reason for this loss is not understood, but it is clear that even the dried sample under vacuum does not represent a static physical environment.

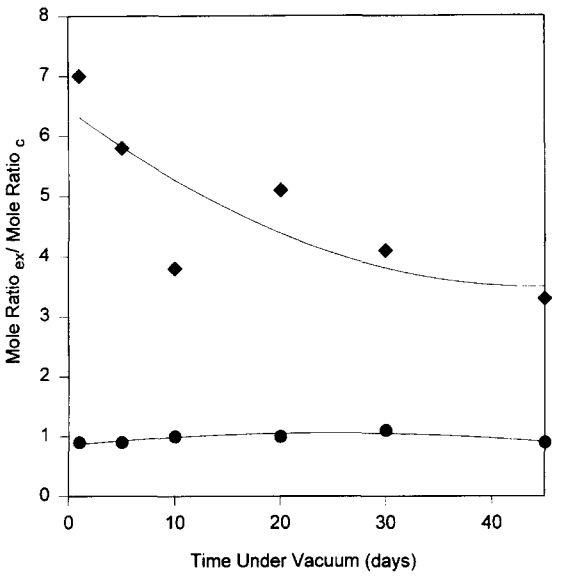

Fig. 6. Elaidic acid production in lyophilized DEPC 9:1 PS liposomes stored for extended periods under vacuum in the dark. Samples in the presence and absence of PLA_2 were allowed to incubate at 22° C for 5 h at 76% RH, then were extracted and measured by analytical thin layer chromatography (◆), or were extracted and measured immediately after removal from vacuum (●). Data presented is the ratio of the experimental mol ratio (Mol $Ratio_{ex}$ = mol elaidic acid/mol PL [+PLA_2]) to the control mol ratio (Mol $Ratio_c$ = mol elaidic acid/mol PL [−PLA_2]).

3.6. FFAs destabilize liposomal membranes

The accumulation of FFAs in the liposomal membranes at low water levels has practical consequences in terms of one of the primary functions of the bilayer, that of a barrier. Fig. 7 shows the increase in membrane permeability caused by the FFAs as measured by retention of the fluorescent solute carboxyfluorescein. DEPC liposomes lyophilized with PLA_2 were incubated at 22° C for 24 h at 47% RH. It can be seen that the increase in elaidic acid in the membrane caused by the enzymatic action of PLA_2 is

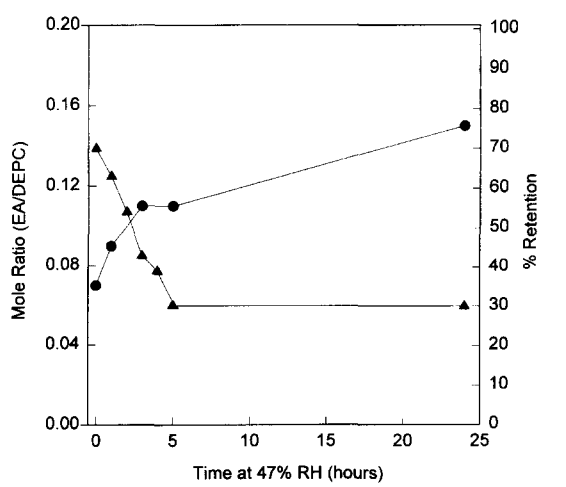

Fig. 7. Elaidic acid production (●) and retention of CF (▲) by DEPC liposomes freeze-dried with PLA_2 during partial rehydration at 47% RH.

mirrored by a decrease in carboxyfluorescein retention over time. Thus, even at low water availability, PLA_2 can induce FFA accumulation in membranes, severely compromising the integrity of the membrane.

4. Discussion

In the fully hydrated system, on which nearly all work regarding PLA_2 has been conducted, the physical state of the lipid substrate has clear effects on the activity of the enzyme. Temperature, phase-state, charge, aggregation level, and curvature strain have all been shown to influence PLA_2 activity [33–45]. The present investigation provides evidence that the physical state of the lipid is important in determining the lipolytic activity of PLA_2 on bilayers in dehydrated and low water systems as well.

First, when liposomes lyophilized with PLA_2 were partially rehydrated in fixed relative humidity chambers, PLA_2 activity increased with incubation at increasing relative humidity. This result is easily explained by the fact that PLA_2 catalyzes a hydrolysis reaction. With more water molecules available to the enzyme, its catalytic activity increases. This argument only holds to a point, however, because PLA_2 was inactive in the fully hydrated system. In the hydrated system, however, calcium was present at only 2 μM. It is likely that this concentration was too far below the mM-range K_d for calcium [15,53,54] to support enzymatic activity. In addition, some form of membrane disruption, such as lateral phase separation would be expected to be necessary in order to activate the enzyme in fully hydrated liposomes.

Second, membrane surface charge also affects PLA_2 activity. Although this was well known in hydrated systems [48–50], it has not been studied in the instance of low water availability. There was a larger accumulation of FFAs in liposomes with higher PS:PC ratios, but water adsorption at a given RH did not increase concomitantly. Thus, the effect of negative charge was not due to attracting more water molecules to the reaction site. This finding correlates well with the work of Jendrasiak and Hasty [47] that showed PS actually has a lower affinity for water than PC. The result also correlates well with other work which has shown negative membrane surface charge to promote PLA_2 activity. Wu and Cho have demonstrated that electrostatic interactions between the membrane surface and PLA_2 are important in PLA_2 regulation [50]. In addition, the enzyme isolated from porcine pancreas has shown a slight preference for anionic phospholipids [48].

Another possible explanation for the charge-induced increase in enzyme activity is that the negatively charged PS created wider spacing between the phospholipid head groups, which might make the *sn-2* fatty acid ester bond more accessible to PLA_2. It has been shown that the average surface area per molecule of a membrane increases with increasing charge in the membrane [52]. Per molecule spacing is also increased with increasing hydration and unsaturation [22,52,55]. Results from the current study indicate that in a spectrum of per molecule surface area, PLA_2 activity increased proportionally with polar head group spacing. This result is supported by the work of Upreti and Jain [56] which shows branched alcohols increased PLA_2 activity in multilamellar vesicles in proportion to their asymmetry and cross-sectional area.

Third, steric hindrance, such as the tight spacing found between lamellae in lyophilized MLVs, either severely inhibits enzyme activity or excludes the enzyme completely. These are difficult possibilities to discriminate between, since PLA_2 is a small protein (14 kDa) (see Ref. [12], for review), so there is some uncertainty as to whether it would fit between the lamellae or not. Thus, three types of evidence, charge, hydration, and steric hindrance, indicate that the physical state of the lipid is of critical importance in the activation level of PLA_2 in dehydrated and low-water systems.

Much about the system, however, remains poorly understood. Liposomes lyophilized in the presence of PLA_2 and stored under vacuum in the dark might be thought to represent a static system. In fact, the enzymatic capacity of PLA_2 diminished during extended storage in this manner. This finding was unexpected, because PLA_2 is an extremely stable enzyme with seven disulfide linkages preserving its native structure [57]. Whether the storage causes damage to the enzyme itself or some alteration in the lipid substrate is not known at this time, but will be the subject of continuing investigation.

Finally, the activity of PLA_2 has clear consequences for the integrity of the membrane. The accumulation of FFAs in the bilayer destabilizes the membrane, increasing its permeability to solutes and decreasing its efficiency as a barrier mechanism.

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