Biochemistry **1997,** *36,* 10551−10557

Changes in Vesicle Morphology Induced by Lateral Phase Separation Modulate Phospholipase A_2 Activity[†]

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Received March 5, 1997; Revised Manuscript Received June 27, 1997[⊗]

ABSTRACT: The action of phospholipase A_2 (PLA_2) toward zwitterionic bilayers is modulated by lateral phase separation of reaction products and substrate. The experiments here address the mechanism of this modulation. PLA_2 is particularly active toward lipid dispersions containing reaction products and substrates in which lateral phase separation has occurred. Here, we study PLA_2 activity in two related model systems: first in a system in which lateral phase separation can be produced *a priori*, and second in a system in which the action of PLA_2 produces sufficient reaction product *in situ* such that lateral phase separation occurs. The dispersions in which lateral phase separation occurs *a priori* form either disk micelles or disk vesicles, not canonical vesicles. When lateral phase separation occurs due to *in situ* PLA_2 activity, there is an abrupt change in vesicle structure and a simultaneous profound increase in catalytic rate. This observation is surprising in light of several reports that vesicles remain intact even when the entire outer monolayer has been hydrolyzed. Membrane curvature and the associated structural defects and dynamic fluctuations in membrane structure have been proposed to modulate PLA_2 activity. The mechanism by which lateral phase separation modulates PLA_2 activity has been unclear. The data presented here indicate that lateral phase separation affects PLA_2 activity by altering membrane curvature and/or inducing defects in the membrane structure.

The physical properties of a membrane may modulate the functions of membrane-bound receptors and enzymes. The activity of extracellular phospholipase A_2 (PLA_2)[1] shows this sensitivity to membrane properties in several ways. Two examples of this sensitivity are as follows: the catalytic behavior varies in a systematic way with vesicle size; the time course is profoundly affected by the reaction temperature relative to the substrate's main phase transition temperature. When venom PLA_2 acts on dipalmitoylphosphatidylcholine (DPPC) large unilamellar vesicles (LUV), the changing membrane composition during hydrolysis affects the catalytic rate, resulting in complex time courses (Burack, *et al.,* 1995). Specifically, PLA_2-catalyzed hydrolysis of gel-state DPPC LUV proceeds slowly until a critical amount of reaction product accumulates. We have previously shown that the accumulation of reaction products results in lateral phase separation of reaction products and substrates, triggering an abrupt increase in catalytic rate (Burack, *et al.,* 1993).

Structural defects and density fluctuations in the bilayer have been proposed to play a prominent role in modulating PLA_2 activity (Wilschut, *et al.,* 1978; Jain & de Haas, 1983; Jain & Jahagirdar, 1985; Menashe, *et al.,* 1986; Bhamidipati & Hamilton, 1989; Sen, *et al.,* 1991). This role is implied by several results. For example, osmotic shock of the vesicular substrate (Lichtenberg, *et al.,* 1986; Upreti & Jain, 1980) or the exogenous addition of some membrane perturbants (e.g., lysophosphatidylcholine) (Jain & de Haas, 1983; Bell & Biltonen, 1992; Zidovetzki, *et al.,* 1992) both eliminate the initial period of slow hydrolysis. We showed that inducing lateral phase separation *a priori* in a mixture of reaction products and substrates also eliminated the initial period of slow hydrolysis and profoundly enhanced PLA_2's catalytic rate. When lateral phase separation occurs, defects in the lipid packing and density fluctuations must occur. PLA_2 may be particularly active at these boundary-associated defects. However, not all conditions which produce compositional or structural heterogeneities are sufficient to eliminate the period of slow hydrolysis. Despite the coexistence of gel and liquid-crystalline domains in the main phase transition region, maximal activity is not instantaneous. Therefore, merely the presence of boundaries between gel and liquid-crystalline domains does not abrogate the effect of lateral phase separation of the reaction products and substrate.

In this work, we show that lateral phase separation of reaction products and substrates is associated with a profound change in vesicle structure. Specifically, when lateral phase separation is induced *a priori*, the lipid dispersions do not form canonical vesicles. Measurements of the bilayer's physico−chemical properties and morphologic studies during *in situ* product accumulation show that lateral phase separation is associated with a catastrophic effect on vesicle structure.

[†] This work was supported by grants from the NIH (GM37658) and NSF (DMB 9005374). W.R.B. was supported by a Medical Scientist Training Grant (GM07267) and A.R.G.D. by an institutional training grant (DK07320) and a National Research Service Award (GM17831).

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[⊗] Abstract published in *Advance ACS Abstracts,* August 15, 1997.

[1] Abbreviations: DPPC, dipalmitoylphosphatidylcholine; LUV, large unilamellar vesicle(s); lyso-PPC,1-palmitoyl-2-lysophosphatidylcholine; MLV, multilamellar vesicle(s); PA, palmitic acid; PLA_2, phospholipase A_2; SUV, small unilamellar vesicle(s); T_m, main phase transition temperature; X_{crit}, critical mole fraction; X_p, mole fraction of reaction product: [PA]/([PA] + [DPPC]) or [lyso-PPC]/([lyso-PPC] + [DPPC]) where [PA] = [lyso-PPC].

S0006-2960(97)00509-6 CCC: $14.00

The implications of these findings for the field are 2-fold. First, these findings are at odds with the often used assumption that the vesicles remain intact throughout the process of hydrolysis, allowing PLA_2 access only to the outer monolayer. This assumption is the basis for the two-dimensional Michaelis−Menten analysis of PLA_2 activity toward vesicle substrates (Berg, *et al.,* 1991; Jain, *et al.,* 1991b). Second, these findings suggest a synthesis of two divergent hypotheses concerning the role of membrane structure in the modulation of PLA_2 activity. Specifically, the manner in which membrane curvature/vesicle morphology modulate PLA_2 has been considered separable from the effects of lateral phase separation. The present findings indicate that the processes may be intimately entwined. We suggest that the role of lateral phase separation may be to induce large-scale changes in vesicle structure and that phospholipids in these new structures are particularly sensitive to hydrolysis by PLA_2.

MATERIALS AND METHODS

Materials. All lipids (except palmitic acid) were from Avanti Polar Lipids (Birmingham, AL). Palmitic acid (PA) and the venom of *Agkistrodon piscivorus piscivorus* were obtained from Sigma Chemical Co. (St. Louis, MO). The basic, monomeric, aspartate-49 isozyme of phospholipase A_2 (PLA_2) was purified from the venom according to published procedures (Maraganore, *et al.,* 1984).

Lipids: Sample Preparation. All lipids were stored at −20 °C in $CHCl_3$, except for 1:1 (mol/mol) mixtures of PA and lyso-PPC which were stored in 1:1 (v/v) $CHCl_3$/MeOH. These lipids formed a flocculent at −20 °C which dissolved when gently heated to 24 °C. To make codispersions, the lipids were mixed in solvent, dried under a stream of N_2, and placed under high vacuum for 8 h. The dried lipids were then hydrated by cycling the temperature 5 times between room temperature and 60 °C and vortexing periodically. The aqueous solvent contained 50 mM KCl with 10 mM HEPES, pH 8.0, for all studies except activity assays, for which the HEPES was omitted. Codispersions with greater than 8 mol % reaction products ($X_p > 0.08$) clarified spontaneously, usually during cooling (see Results). Extruded large unilamellar vesicles (LUV) were prepared from MLV and characterized as previously described (Burack, *et al.,* 1993). Extrusion of codispersion with $X_p > 0.08$ codispersions did not alter the thermotropic properties as determined by differential scanning calorimetry. This result indicated that the composition of the lipid dispersion was not altered upon extrusion. Small unilamellar vesicles (SUV) were prepared by sonication and stored above the T_m until used. All other lipid preparations were temperature cycled (25 °C to approximately 60 °C) 3 times immediately before use and were cooled to the reaction temperature.

Activity Assays and 90° Light Scatter. Light scatter and product accumulation, as monitored with a Radiometer Autoburette in the pH-stat mode, were determined as previously described (Burack, *et al.,* 1993). The duration of the initial period of slow hydrolysis varies with each preparation of vesicles. To perform experiments of convenient durations, the concentration of calcium was altered. The final concentrations are given in each figure legend.

Electron Microscopy. For negative-stain electron microscopy, a 100 μM solution of lipid was incubated with a washed carbon film for 30 s. To stain, the carbon film was then incubated for 1 min with an isosmotic solution which included 25 mM uranyl formate. The films were allowed to dry at room temperature for approximately 5 min and were promptly examined. Preparations of samples by rapid freezing, freeze−fracture, and replica-coating are essentially as described by Heuser (1983).

^{31}P-NMR. The ^{31}P-NMR data were collected using a Varian 500 Unity Plus spectrometer operating at 202.3 MHZ with the following instrument settings: sweep width, 50 kHz; 90° pulse width, 20−26 μs; acquisition time, 50−550 ms; time between pulses, 1.2 s; number of transients, 500−2944. An exponential multiplication corresponding to a line broadening of 10−200 Hz was applied to the accumulated free induction decays prior to Fourier transformation. The lipid samples were prepared as described above except that the buffer included 20% (v/v) D_2O. SUV of egg phosphatidylcholine at 30 °C were used to define 0ppm. Other experimental details are given in the figure legends.

RESULTS

We use two related model systems with which to study the effect of membrane structure on PLA_2's catalytic rate (Burack, *et al.*, 1993). In the first system, the substrate is a zwitterionic lipid (DPPC) presented as a large unilamellar vesicle in the gel state. Under these conditions, there is a period of slow product accumulation (the "lag" period) which ends abruptly with a sudden acceleration. The lag period can last several hours and ends when the catalytic rate increases as much as 3 orders of magnitude in just a few seconds. Using this system, we have previously shown that the abrupt increase in catalytic rate is concomitant with an abrupt redistribution of a fluorescent fatty acid analog, which we interpreted to indicate lateral phase separation of lipid components. In the second system, the substrate is DPPC codispersed with varying mole fractions of a 1:1 mix of the lyso-PPC and palmitic acid, the reaction products. A partial phase diagram was prepared for this mixture indicating that at $X_p \geq 0.085$ compositional phase separation occurs. The lag period decreased monotonically for codispersions with increasing fractions of reaction products, and the lag was abolished for $X_p = 0.085$. This last result indicates that lateral phase separation is the cause, rather than the effect, of rapid hydrolysis (Burack, *et al.*, 1993). Although the lipid compositions in these two model systems are similar, the overall structures of the lipid aggregates are expected to be distinct; the *in situ* accumulation of reaction product will occur predominantly in the outer monolayer, while codispersions of reaction product and substrate will result in a less asymmetric distribution.

System 1: Large-Scale Changes in LUV Due to the Action of PLA_2 in Situ. Product accumulation and 90° light scatter were recorded simultaneously using gel-state DPPC LUV as the substrate (data not shown). The intensity of the scattered light is constant until the abrupt increase in catalytic rate, when the light scatter drops precipitously to 70% of its original value. Similar results were obtained above the main phase transition temperature. While it is not possible to assign a quantitative meaning to these changes in the scattering intensity, these data imply that there is a large change in the overall size, shape, and/or number density of the vesicles concomitant with the abrupt acceleration of catalysis.

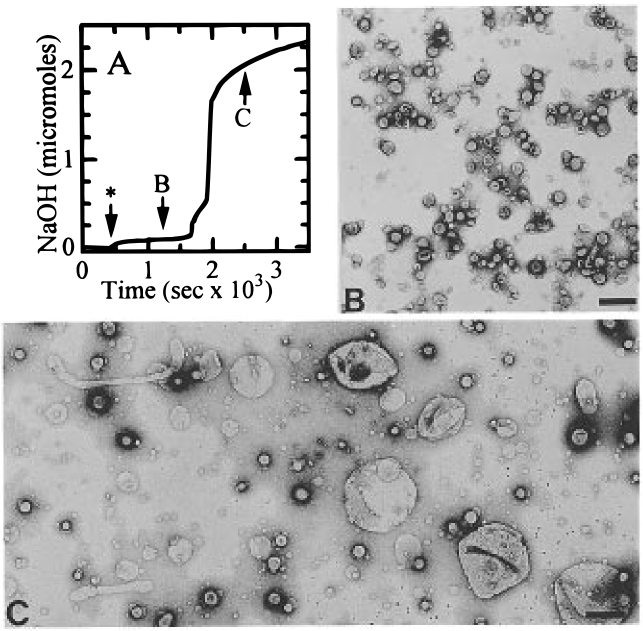

FIGURE 1: Hydrolysis time course of gel-state DPPC LUV and negative-stain electron micrographs obtained prior to and after extensive hydrolysis of gel-state DPPC LUV. The reaction was initiated with the addition of PLA_2 (*). Fifty microliter aliquots were removed at the times marked as B and C. These samples were immediately diluted to 100 μM lipid with the reaction solution and prepared for electron microscopy. The corresponding micrographs are shown in panels B and C and are on the same scale (bars are 4000 Å). Conditions: 50 mM KCl, 10 μM EGTA, 20 μM $CaCl_2$, 5 mM DPPC LUV, 1 μM PLA_2, 36.5 °C.

To characterize changes in the vesicles during catalysis, negative-stain electron micrographs were obtained of substrate vesicles during the lag period and shortly after the abrupt catalytic acceleration (Figure 1). The time and extent of hydrolysis for each micrograph are indicated in Figure 1A by the arrowheads. The lipid structures derived from the lag period (Figure 1B) are a homogeneous collection of 500−1000 Å diameter vesicles, a population which is indistinguishable from LUV which have not been exposed to PLA_2. However, the structures obtained from a time shortly after the acceleration (Figure 1C) show a variety of forms. The structures in Figure 1C include vesicles which are smaller and others which are far larger than the original vesicles. These data show that there is a profound change in vesicle structure associated with the abrupt acceleration.

Vesicle structure during hydrolysis was further investigated using ^{31}P-NMR. Figure 2 shows parallel ^{31}P-NMR and proton release time courses using the same preparations of PLA_2 and LUV. Using this preparation, the initial period of slow hydrolysis ranged between 3 and 5 h. A typical proton release time course is shown. The multiphasic proton release time course has been previously observed, and we have attributed the complexity to nonequilibrium heterogeneous distributions of reaction products in the membrane (Burack, *et al.*, 1994). For the NMR studies, PLA_2 was added to the vesicles, and data were acquired continuously, each spectrum representing 1 h. The initial three spectra are typical of gel-state LUV. Corresponding to the time of the abrupt catalytic acceleration, a narrow component in the spectra appears. This component ceases to grow approximately when the rapid phase of proton release ends.

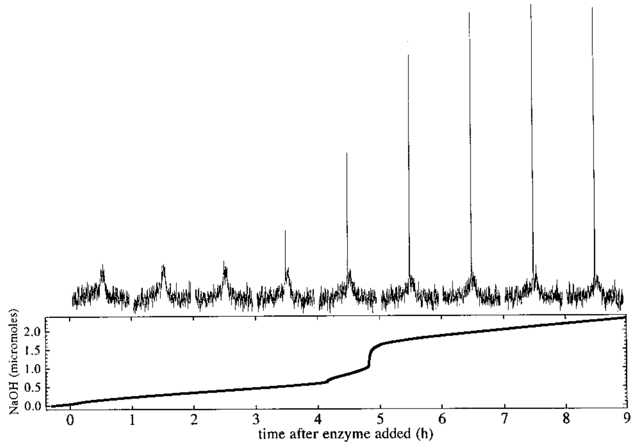

FIGURE 2: Time courses of PLA_2 activity toward DPPC LUV assessed in parallel by ^{31}P-NMR spectroscopy and proton release. The reactions were initiated by the addition of PLA_2. Reaction conditions for both experiments are 36.5 °C, 1 μM PLA_2, 5 mM DPPC, 50 mM KCl, 20 μM $CaCl_2$, 10 μM EDTA, 20% D_2O. For ^{31}P-NMR, the reaction includes 10 mM HEPES, pH 8.0. The data were acquired using 100 Hz line broadening and 2944 transients/spectrum, requiring 1 h/spectrum. The free induction decays were proton decoupled.

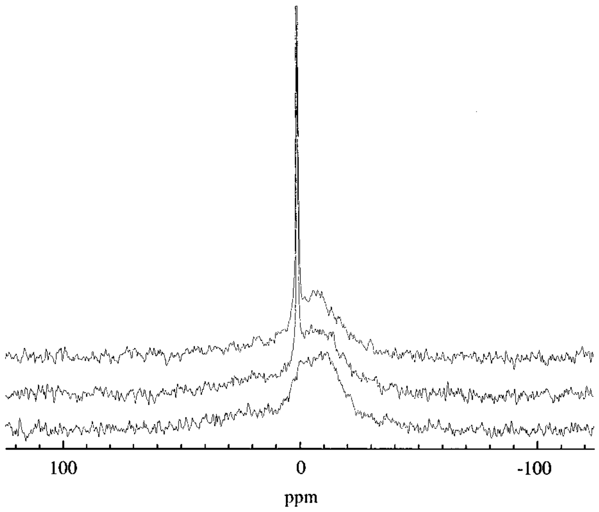

FIGURE 3: Representative ^{31}P-NMR spectra of DPPC LUV during hydrolysis by PLA_2. Reactions conditions are as in Figure 2 except 5 μM PLA_2, 25 mM DPPC, and 1472 transients/spectrum, requiring 30 min/spectrum. The spectra were obtained 0−0.5 (bottom), 6−6.5 (middle), and 11−11.5 (top) h after addition of enzyme. The peak of the last spectrum is off-scale, and the actual peak intensity is twice the intensity shown here.

To allow a more detailed study of lipid structure during product accumulation *in situ*, the parallel ^{31}P-NMR and proton release time courses were repeated using a higher lipid concentration (Figure 3). The concentration of PLA_2 was increased to maintain the same enzyme:lipid ratio used in Figure 2. Three spectra are shown, each corresponding to a specific period of the time course: an initial spectrum acquired shortly after addition of PLA_2 but before the abrupt catalytic acceleration, a spectrum acquired during the period of rapid hydrolysis (as indicated by the appearance of the isotropic component), and a final spectrum acquired once hydrolysis has slowed and the spectra are no longer changing perceptibly. Despite the obvious growth of a spectral component reflecting an isotropic phospholipid structure, the characteristic spectrum of anisotropic phospholipid structures is present at all times. In the final spectrum, the integrated intensity of the anisotropic component is 81% of that in the initial spectrum.

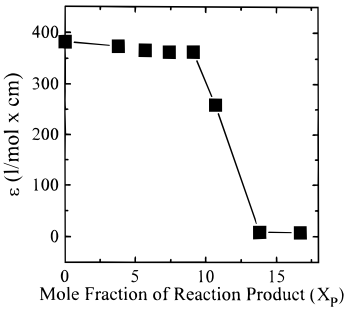

FIGURE 4: Effect of the mole fraction of reaction product on the extinction coefficient of DPPC−reaction product codispersions. The absorbance of the extruded codispersions was determined at 500 nm. Assay conditions were 50 mM KCl, 10 mM HEPES, pH 8, 5 μM $CaCl_2$, 3 mM lipid, 24 °C.

System 2: Macromolecular Structure of Codispersions. Lateral phase separation has been characterized in codispersions of substrate and reaction products (Burack, *et al.*, 1993). While DPPC/reaction product codispersions with $X_p < 0.08$ are plainly turbid to the eye, codispersions with $X_p \geq 0.08$ are transparent. The effect of X_p on the optical density is shown in Figure 4. The structures with low optical density (formed by hydrating and cycling the codispersions through the T_m several times) were further characterized using electron microscopy and ^{31}P-NMR.

Figure 5 shows electron micrographs of the structures formed by hydrating and temperature cycling ternary codispersions with $X_p = 0.16$. The preparation was dried on a carbon film and visualized with a negative stain (5A). Because drying may introduce artifactual morphologies, samples were also prepared by rapid freezing and fracturing, and metal replicas were made by both fixed-angle (5B) and rotary shadowing (5C). All three preparations show a homogeneous suspension of discoidal particles with diameters of about 500 Å and widths of about 100 Å. The negative-stained images (5A) are strikingly noncentrosymmetric: there is an obvious “cut-edge” which does not stain. The discoidal structures seen by fixed-angle shadowing of freeze-replicas (5B) appear centrosymmetric. A homogeneous population of disklike structures also appears in the rotary-shadowed freeze-replicas (5C). The symmetry of these particles is not obvious and may be affected by the orientation of the particles relative to the fracture plane. No evidence of hexII or other extended phases was seen. The resolution of our freeze-fracture data is not sufficient to determine whether the discoidal particles are one bilayer thick (i.e., disk micelles) or two bilayers thick (i.e., disk vesicles). The structures produced by fused DPPC LUV, extruded DPPC LUV, and sonicated DPPC SUV all have distinctive micrographic appearances and cannot be confused with the disklike particles spontaneously formed by these codispersions.

^{31}P-NMR was used to further characterize these disklike particles. Figure 6 shows the effect of varying X_p on the ^{31}P-NMR spectrum of ternary codispersions at 30 °C. When $X_p \leq 0.08$, the spectra are the powder patterns typical of multilamellar vesicles (6A−C). However, increasing X_p from 0.077 to 0.1 caused the spectrum to become far narrower and isotropic (compare 6C and 6D.) In Figure 7, the ^{31}P-NMR spectra of DPPC small unilamellar vesicles (SUV), ternary codispersions with $X_p = 0.17$, and lyso-PPC micelles are compared. The spectra of ternary codispersions and SUV are similar. Barring fast exchange of lipids between structures, the coexistence of vesicular and micellar phospholipid in the ternary codispersion would produce a multicomponent spectrum; due to the extremely narrow and intense spectrum of the micelles, we calculate that <1% contamination with lyso-PPC micelles would be readily evident. The similarity of the spectra to those of SUV is consistent with the electron microscopy results which showed that the codispersions spontaneously form particles with sizes similar to those of SUV.

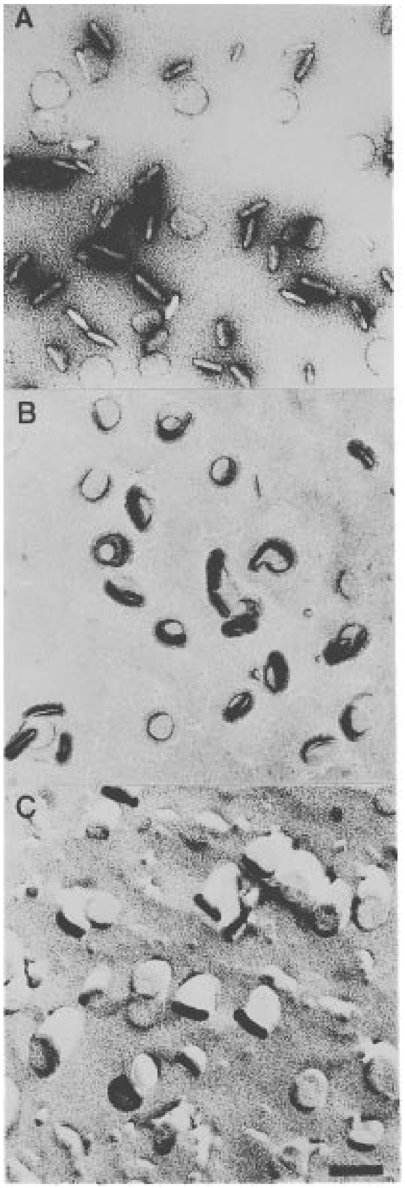

FIGURE 5: Electron micrographs of a codispersion of DPPC with $X_p = 0.16$. Three techniques were used to prepare samples: dried on carbon film and negative-stained (A); freeze−fracture followed by either rotary shadowing (B) or fixed-angle shadowing (C). The scale bar is 500 Å.

DISCUSSION

Lateral phase separation has been proposed to modulate the activity of a secretory and a venom PLA_2 (Jain, *et al.*, 1989; Burack, *et al.*, 1993). The mechanisms underlying this modulation remain unclear. Using monolayer systems, Grainger and co-workers provided striking evidence that PLA_2-catalyzed hydrolysis induces compositional heteroge-

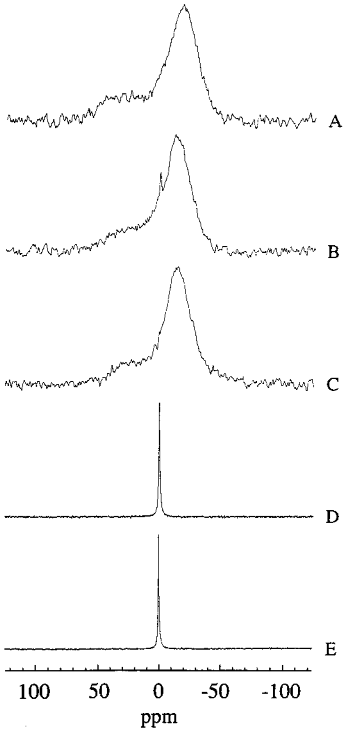

FIGURE 6: Effect of X_p on the ^{31}P-NMR spectrum of DPPC LUV. The mole fractions of reaction products are 0.0 (A), 0.05 (B), 0.077 (C), 0.10 (D), and 0.17 (E). The spectra were obtained at 30 °C with 15 mM phospholipid. Spectra A−C: 2000 transients, 200 Hz line broadening. Spectra D−E: 500 transients, 20 Hz line broadening. The free induction decays were not proton-decoupled.

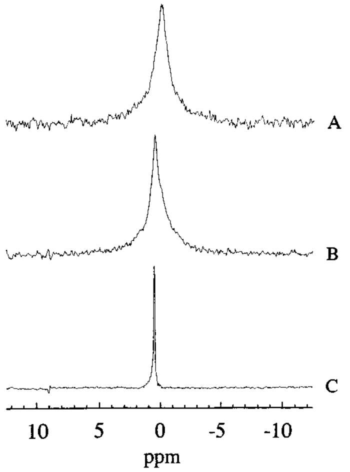

FIGURE 7: Comparison of the ^{31}P-NMR spectra of DPPC SUV (A), ternary codispersions with $X_p = 0.17$ (B), and a lyso-PPC dispersion (C) The temperature was 30 °C. Spectra A and B: 640 transients, 15 mM lipid. Spectrum C: 1000 transients, 1 mM. For all spectra, the line broadening was 10 Hz, and the free induction decays were not proton-decoupled.

neities (1989). Use of binary lipid systems as substrates has provided some evidence that mixing properties of substrates modulate PLA$_2$ activity (Gabriel, *et al.*, 1987). Smaby *et al.* argue that compositional heterogeneities, but not complete lateral phase separation, modulate carboxy-ester lipase activity toward monolayer substrates (1994). Although there appears to be some role for lateral phase separation, or compositional heterogeneities, in modulating PLA$_2$ activity toward bilayer substrates, roles for membrane curvature, "defects", and dynamic fluctuations of lipid packing have also frequently been invoked (op den Kamp, *et al.*, 1974; Grainger, *et al.*, 1989; Sen, *et al.*, 1991; Zidovetzki, *et al.*, 1992; Bell & Biltonen, 1992; Jain & de Haas, 1983; Upreti & Jain, 1980; Jain & Berg, 1989; Bell, *et al.*, 1995, Bell, *et al.*, 1996; Sheffield, *et al.*, 1995).

Vesicle Structure Changes during Hydrolysis. Although LUV have been only rarely used as substrates, the integrity of SUV during hydrolysis both above and below the phase transition has been examined by others. Three studies employing different techniques have been interpreted to show that SUV structure is preserved even when the entire outer monolayer has been hydrolyzed. These studies examined permeability to paramagnetic ions (Jones & Hunt, 1985), encapsulation of carboxyfluorescein (Wilschut, *et al.*, 1979), and dequenching of a membrane-bound fluorescent probe (Jain, *et al.*, 1986). Furthermore, while the two reaction products, lyso-PPC and PA, can each induce DPPC to form nonlamellar phases, a 1:1 mix of these products is reported to form lamellar structures (Jain, *et al.*, 1980). In earlier work, we noted that 80% of an entrapped dye leaked from DPPC LUV at the time of the abrupt catalytic acceleration, and 20% remained encapsulated even after prolonged hydrolysis (Burack, *et al.*, 1993). Nevertheless, given the literature implying that vesicles remained intact throughout hydrolysis, we previously suggested that vesicle structure is maintained during the hydrolysis of DPPC LUV. Here, we report a large and abrupt change in light scatter intensity concomitant with the acceleration in catalytic rate, indicating that some property of the lipid structures is profoundly altered. Furthermore, the electron micrographs show that the *in situ* action of PLA$_2$ produces a heterogeneous mix of lipid structures, including very large structures which must have resulted from vesicle fusion. The vast majority of the structures seen at late times are clearly distinguishable from the initial LUV. The ^{31}P-NMR data show that even after long periods of hydrolysis the bulk of the phospholipid remains in structures which give rise to lamellar powder patterns. These spectra suggest that the resulting structures are not significantly smaller than the original vesicles. The action of PLA$_2$ *in situ* also produces some structure with an isotropic NMR spectrum having a full-width at half-height similar to that of lyso-PPC micelles. Therefore, vesicular and micellar (or some other highly curved) states apparently coexist. These data show that while the action of PLA$_2$ does not abolish vesicle structure, hydrolysis produces new types of phospholipid aggregates.

Some investigators have used the purported integrity of vesicle structure throughout the time course of hydrolysis to indicate that the entire outer monolayer can be comprised of reaction products while still maintaining vesicle structure (Jain, *et al.*, 1986). This assumption about vesicle stability during hydrolysis forms the basis for extensive modeling of PLA$_2$ activity as a simple Michaelis−Menten system (Jain & Berg, 1989; Jain, *et al.*, 1991a,b; Berg, *et al.*, 1991; Ghomashchi, *et al.*, 1991). Since all product was assumed to reside in the outer monolayer and the vesicles were presumed stable, the hydrolysis could be described as occurring in a two-dimensional solution, where the amount of product accumulated indicated the concentration of product present in that solution. The parameters of this Michaelis−Menten model are derived from experiments in which the substrate is SUV composed entirely of anionic phospholipids. This anionic SUV substrate is very different from the zwitterionic LUV substrate used here and could behave differently when product accumulates. However, fatty acid translocation from the outer to the inner monolayer during hydrolysis occurs, showing that the assumption of vesicle stability is not strictly correct (Bhamidipati & Hamilton, 1989; Alix & Woodbury, 1997). Furthermore, our data indicate that it is grossly inappropriate to apply the assumption to PLA_2 action on gel-state DPPC LUV because vesicle structure is clearly perturbed by the accumulation of reaction product. These findings suggest that assumptions about vesicle stability should be rigorously tested to determine whether it is appropriate to model phospholipase activity toward a vesicular substrate as a Michaelis−Menten system.

Structure of Codispersions with $X_p > X_{crit}$. In previous work, we used codispersions of substrates and reaction products to show that lateral phase separation is the cause rather than the effect of accelerated catalysis. Specifically, for fully hydrated codispersions of DPPC and 1:1 lyso-PPC/PA, the X_{crit} for lateral phase separation in the gel state is about 0.08, and the initial period of slow catalysis is eliminated when $X_{\mathrm{p}} > X_{\mathrm{crit}}$ (Burack, *et al.*, 1993). Here, we show that when $X_{\mathrm{p}} > X_{\mathrm{crit}}$, the codispersions have low turbidity, are comprised of disklike particles with diameters of ∼500 Å and thickness of ∼100 Å, and have ^{31}P-NMR spectra that appear similar to the spectra of SUV. Therefore, these structures are also clearly different from those formed by *in situ* accumulation of reaction products in DPPC LUV. What the two systems have in common is that the presence of reaction products profoundly disturbs vesicle structure.

Implications of Global Structural Changes Due to Product Accumulation for the Activation Process. Membrane defects, fluctuations, and curvature have been proposed to modulate PLA_2 activity. The evidence for these mechanisms of modulation is all of the correlative type presented here. For example, studies of sonicated SUV have shown that the enzyme is active toward some highly curved bilayers without a lag period (op den Kamp, *et al.*, 1975; Menashe, *et al.*, 1986; Gheriani-Gruszka, *et al.*, 1988). Osmotic shock of DPPC LUV which is presumed to cause membrane defects and/or fluctuations also results in immediate activity (Lichtenberg, *et al.*, 1986; Upreti & Jain, 1980). Sen and Hui have observed PLA_2 activity as a function of the mole fraction of a phospholipid which has a tendency toward forming the HexII phase (1991). These investigators concluded that maximal activity was correlated with compositions just lower than that which induces a lamellar/HexII phase transition. They proposed that these conditions resulted in increased bilayer stress and maximal fluctuations in lipid packing. Sheffield et al. (1995) used the hydration-sensitive, membrane-associated fluorescent probes, prodan and lauradan, to study the correlation between changes in membrane structure and PLA_2 activity. The fluorescence of these probes decreased abruptly when the catalytic rate increased. The decrease in fluorescence, indicating increased hydration, is thought to indicate increased membrane curvature. Therefore, these authors also suggested that product accumulation, especially the accumulation of lyso-PPC, is associated with the formation of regions with high positive curvature. All of these interpretations could be applied to our data.

The kinds of effects described here as modulators of PLA_2 probably apply to other enzymes as well. Recently, Basanez *et al.* showed that phospholipase C (PLC) exhibits anomalous kinetics very similar to those seen with PLA_2 (1996). Furthermore, the abrupt catalytic acceleration was accompanied by a large increase in turbidity. These authors suggest that the accumulation of a critical amount of reaction product, diacylglycerol, induces lateral phase separation, perturbs the vesicle structure, and drives vesicle aggregation and fusion. The authors state “whether [lateral phase separation or vesicle aggregation/fusion] is the primary agent of phospholipase C activation cannot be ascertained, mainly because, in our system, both appear to occur simultaneously”. Therefore, the mechanisms by which PLA_2 and PLC are modulated by membrane properties are probably similar. However, we suggest that it is unnecessary to distinguish between lateral phase separation and perturbation of vesicle structure by reaction products: our data suggest that these processes are intimately coupled.

We present findings in two related but distinct model systems suggesting that morphologic constraints play a key role in modulating PLA_2 activity: first, profound structural changes are associated with the onset of rapid hydrolysis; second, structures toward which activity is immediate are not canonical vesicles. These data cannot unequivocally demonstrate that changes in vesicle morphology are the cause (rather than the effect) of rapid product accumulation *in situ*. However, there is a substantial body of literature using the same kind of logic suggesting that increased membrane curvature, defects, and the fluctuations inferred to accompany defects all enhance PLA_2 activity (for example, Zidovetzki, *et al.*, 1992; Sen, *et al.*, 1991; Upreti & Jain, 1980; Wilschut, *et al.*, 1978). All of these effects/properties are predicted if profound morphologic rearrangements are responsible for modulating PLA_2 activity. In this way, a morphologic model provides both a synthesis of the literature and a physical picture of the membrane toward which PLA_2 is active. For example, detailed knowledge of the effect of lateral phase separation in *codispersions* allows the suggestion of a specific structure as the defect responsible for activation: the highly curved edges of the discoidal particles. Regardless of the precise nature of the structures which are formed when lateral phase separation occurs due to the *in situ* action of PLA_2 , it is clear that the phase separation of reaction products and substrate is associated with global structural rearrangements. The structures formed during hydrolysis are likely to be heterogeneous and will include morphologic intermediates which may be important determinants of activity. Sackmann and Feder have presented a general theoretical model for coupling between lateral phase separation and changes in vesicle morphology (1995). The intimate coupling between vesicle morphology and lateral phase separation suggests a mechanism for the profound modulatory effect that lateral phase separation exerts on PLA_2 activity.

ACKNOWLEDGMENT

We thank Dr. Thomas Hønger of the Danish Technical University for many stimulating discussions.

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BI970509F