# Role of Lateral Phase Separation in the Modulation of Phospholipase $A_2$ Activity†

W. Richard Burack, Qiang Yuan, and Rodney L. Biltonen*

*Departments of Biochemistry and Pharmacology, University of Virginia Health Sciences Center, Charlottesville, Virginia 22908*

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ABSTRACT: Phospholipase $A_2$-catalyzed hydrolysis of phosphatidylcholine large unilamellar vesicles is characterized by a period of slow hydrolysis followed by a rapid increase in the rate of hydrolysis. The temporal relationship between the burst of $PLA_2$ activity and the lateral distribution of substrate and product lipids was examined by simultaneously recording product accumulation and the fluorescence of 1-pyrenyldecanoate, a fatty acid derivative sensitive to lipid distribution and lateral diffusion. The excimer: monomer ratio of the probe changes slowly prior to the burst in activity and then abruptly at the time of the burst. A partial phase diagram for the ternary codispersion of substrate and products (dipalmitoylphosphatidylcholine and 1:1 monopalmitoylphosphatidylcholine/palmitic acid) was constructed by differential scanning calorimetry and suggests gel/gel immiscibility in this system. Thus, the changes in pyrene fluorescence during the time course of hydrolysis appear to be due to lateral phase separation. The critical mole fraction of product both for lateral phase separation in the gel state and for elimination of the lag phase is approximately 0.083. The simultaneous recordings of $PLA_2$ activity and pyrene fluorescence show that the lateral rearrangement of lipids begins prior to and continues during the rapid activation process of $PLA_2$. Two possible effects of lateral phase separation are that concentration of the protein in the product-rich regions promotes putative dimerization or that formation of phase interface regions promotes enzyme activation.

The extracellular phospholipase $A_2$ has been extensively studied as a model for assessing the effect of membrane structure on enzyme activity. There are three salient characteristics of $PLA_2$ activity on certain zwitterionic bilayer vesicles which suggest that this is a particularly useful model system. First, enzyme activity exhibits a lag phase which ends abruptly with a "burst" of activity. Activity can increase as much as 3 orders of magnitude over times as short as several seconds following a lag of scores of minutes. Second, the lag time as a function of temperature shows a pronounced minimum in the gel–liquid-crystalline phase transition region of the substrate. Third, the lag time can be altered by addition of a variety of hydrophobic or amphipathic molecules, including one of the products of the $PLA_2$-catalyzed hydrolysis of zwitterionic phospholipid.

The critical activation process has been modeled as both equilibrium and slow kinetic processes (Jain & Berg, 1989; Bell & Biltonen, 1992). The equilibrium models focus on the affinity of phospholipase $A_2$ for zwitterionic membranes and a putative change in affinity that occurs as product accumulates within the membrane (Apitz-Castro et al., 1982). However, the inability of high concentrations of substrate to support high initial activities encouraged the creation of kinetic models (Verger et al., 1973; Romero et al., 1987). These models invoke a number of slow processes, the rates of which can be enhanced by the presence of product. While these two classes of models differ substantially, both imply the existence of a critical role of membrane composition to rationalize the abrupt burst in activity.

In the lipid system examined in this work, $PLA_2$ activity is sensitive to both the membrane's physical structure (as demonstrated by the temperature effect on the lag time) and the composition. These two sensitivities may be closely related. Noting that substantial activity was only observed near the substrate's $T_m$, Op den Kamp et al. (1974) first proposed that the coexistence of structurally distinct (gel and liquid-crystalline) regions is important. Later, Apitz-Castro et al. (1982) found that there appears to be a fixed mole fraction of reaction products at the time of the burst in enzyme activity. These authors suggested that because certain lipid species mix poorly with one another, domains enriched in one species (in this case, reaction products) may form. This process, referred to as lateral phase separation, would also result in the coexistence of structurally distinct regions. The dual sensitivity of $PLA_2$ activity to temperature and composition suggests that lateral phase separation might play a role in the regulation of $PLA_2$. Jain et al. (1989) explicitly stated this hypothesis and reported observations consistent with a central role of lateral phase separation in the burst of $PLA_2$ activity.

To better understand the possible role of lateral phase separation in modulation of $PLA_2$ activity, we developed a method to monitor lateral rearrangement of lipid during the hydrolysis of lipid bilayer membrane by $PLA_2$. We use this methodology to examine the temporal correlation between lateral phase separation and the activity of $PLA_2$. In addition to these kinetic measurements, the equilibrium miscibility of products and substrate was characterized.

## MATERIALS AND METHODS

Dipalmitoylphosphatidylcholine (DPPC) and L-α-monopalmitoylphosphatidylcholine (lyso-PC) were obtained from Avanti Polar Lipids (Birmingham, AL). These reagents were found to be pure by thin-layer chromatography and were used without further purification. Palmitic acid was obtained from Sigma Chemical Co. (St. Louis, MO). Palmitic acid (PA) and lyso-PC were stored in 1:1 $CH_3Cl$/MeOH. 1-Pyrenyldecanoic acid, 1,2-bis(1-pyrenyldecanoyl)-*sn*-glycero-3-phosphocholine, 8-aminonaphthalene-1,3,6-trisulfonic acid (ANTS), and p-xylylnebis(pyridinium bromide) (DPX) were obtained

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* To whom correspondence should be addressed at the Department of Pharmacology, University of Virginia Health Sciences Center.

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from Molecular Probes, Inc. (Eugene, OR), and were used without further purification.

Venom of *Agkistrodon piscivorus piscivorus* was obtained from Sigma Chemical Co. 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). The resulting purified $PLA_2$, App-D49, was dissolved in 50 mM KCl. The enzyme concentration was determined from its absorption at 280 nm using $E^{1\%}_{280nm} = 22.0$.

All lipids were stored in organic solvent at −20 °C. The stock of palmitic acid was heated gently in warm tap water until clarification. Lipids were then mixed and dried under a stream of $N_2$ (from $CHCl_3$ for DPPC or 1:1 $CHCl_3$/MeOH for ternary mixtures containing monopalmitoyl-PC and palmitic acid) and lyophilized for 16 h or more. Dried lipid was dispersed in buffer (10 μM $CaCl_2$, 50 mM KCl, and 1 mM $NaN_3$) at 55–60 °C to form a dispersion of multilamellar vesicles (MLV). The buffer included 10 mM sodium borate, pH 8.0, for the calorimetry and fluorescence experiments or 10 mM HEPES, pH 8.0, where noted in the figure legends. The dispersion was frozen (−70 °C) and thawed (55 °C) at least 3 times. Large unilamellar vesicles (LUV) were prepared by extruding the MLV dispersion at least 10 times through two 0.1-μm pore size Nucleopore polycarbonate filters using a high-pressure extrusion device from Lipex Biomembranes Inc., Vancouver, British Columbia (Hope et al., 1985). The temperature of the lipid dispersion was maintained above the phase transition temperature of the lipid components (usually 50 °C) during extrusion. The resulting LUV preparations were characterized by quasi-elastic light scattering (QLS) using a Nicomp Model 370 submicron particle sizer. The size distribution for most preparations fit well to a Gaussian with an average diameter of 100–120 nm and standard deviations varying from 20 to 40 nm.

Differential scanning calorimetry (DSC) was performed on an instrument produced by Hart Scientific (Provo, UT). One-milliliter samples were scanned at 20 °C/h. Appropriate base lines have been subtracted from the excess heat capacity curves. Onset and offset temperatures for the main phase transition were determined by the method of Mabrey and Sturtevant (1976). No correction has been made for the time response of the instrument.

All fluorescence measurements were made with a T-format SLM 8000C in the slow analog mode. For measurements of pyrene excimer:monomer ratio, the excitation wavelength was 346 nm, and the emission wavelengths were 398 (monomer) and 500 nm (excimer). The intensities at 398 and 500 nm were monitored simultaneously. The excimer fluorescence was monitored at 500 nm to avoid signals arising from the shoulder of the relatively intense monomer fluorescence. All slit widths were 4 nm. Samples were stirred continuously. The temperature within the cuvette was measured with a calibrated thermistor inserted directly into the sample. Specific conditions are described in the figure legends.

For permeability studies, 10 mM DPPC was dispersed in the presence of the dyes ANTS and DPX (50 mM each, pH 8.0). Vesicles were extruded at 50 °C and then rapidly chilled to 4 °C. Untrapped dye was removed by gel filtration on Sephacryl-200 (15 × 2 cm) at room temperature, eluting with 100 mM KCl. The hydrolysis reaction mixture contained 300 μM DPPC, 100 mM KCl, 5 μM $CaCl_2$, and 450 nM AppD49 at 35 °C. Leakage of dye from the vesicles, 90° light scatter, and product accumulation were monitored simultaneously. The excitation and emission wavelengths were 335 and 515 nm, respectively; 90° light scattering was also monitored at 335 nm.

Negative-stain electron microscopy was performed as described in Gonias et al. (1988) except that no glutaraldehyde fixation was employed.

The time course of product accumulation was monitored by a Radiometer Autoburette in the pH-stat mode. A capillary tube fitted to the buret tip, a small-diameter combination electrode, and a thermistor were placed directly into the cuvette in the fluorometer. The autotitration unit has been modified to allow data to be recorded digitally and synchronously with the fluorescence data. Titrations were recorded on a personal computer using a Metrabyte DASH8 A/D converter. The data were recorded as the time (within 50 ms) of each 0.1-μL injection of titrant. The titrant was 2.5 mM NaOH in a 50 mM KCl solution. The reaction volume was 2.5 mL. The reactions were unbuffered, and the autotitrator was set to maintain the pH at 8.00.

## RESULTS

We chose a pyrene-labeled fatty acid as an optical probe for the putative lateral phase separation of reaction products and substrate. This fluorescent probe has two characteristics which make it useful in these experiments. First, it is a fatty acid derivative and will likely partition between domains enriched in substrate and reaction products much as the reaction product, palmitic acid. Second, the emission spectrum of the pyrene fluorophore is sensitive to the local concentration and lateral mobility of the probe molecule. The excited-state pyrene molecule has a well-defined, structured emission spectrum with a maximum at 398 nm. If an excited-state pyrene molecule encounters a ground-state pyrene molecule, an excited-state complex can be formed. This excited-state dimer, called an excimer, has a distinct emission spectrum with a maximum at 460 nm.

Assuming that the ratio of the excimer fluorescence intensity to the monomer fluorescence intensity ($E/M$) reflects the aggregation of pyrenyldecanoate and that this probe distributes preferentially into domains enriched in product, then an increase in the $E/M$ ratio is expected when phase separation occurs. This increase is due to the concentrating of the pyrenyldecanoate probe in the minor of the two compositionally distinct phases. There will be some mole fraction of phase-separated material which produces a maximum $E/M$ ratio. This model for the behavior of pyrenyldecanoate predicts an excursion in $E/M$ during $PLA_2$-catalyzed hydrolysis of LUV if the products and substrates phase-separate.

The hydrolysis of large unilamellar vesicles (LUV) composed of DPPC and 1-pyrenyldecanoic acid (500:1) was catalyzed by the aspartate-49 $PLA_2$ from *Agkistrodon piscivorus piscivorus*. In each panel of Figure 1 is a pair of simultaneously recorded time courses of the $E/M$ and product accumulation. The enzyme:vesicle ratios are about 100:1, 50:1, and 25:1 in panels A, B, and C, respectively.[1] The maximum in $E/M$ is obvious. The maximum value of $E/M$ was independent of the concentration of $PLA_2$. The initial and final fluorescence intensities for both the excimer and monomer were each nearly the same, resulting in very similar initial and final values for $E/M$. (The small decrease in $E/M$ at the start of each reaction is due to temperature equilibration. Once equilibrated, the temperature was maintained at 37.3

[1] For reasons explained below, these studies are conducted at a low concentration of $CaCl_2$ (5–10 μM). This concentration is well below that which results in half-maximal activity under similar conditions (Lathrop & Biltonen, 1992). To observe reactions on a reasonable time scale, relatively high concentrations of $PLA_2$ were used. At high $PLA_2$:vesicle ratios, Bell and Biltonen (1992) observed that the lag time is only weakly dependent on $PLA_2$ concentration.

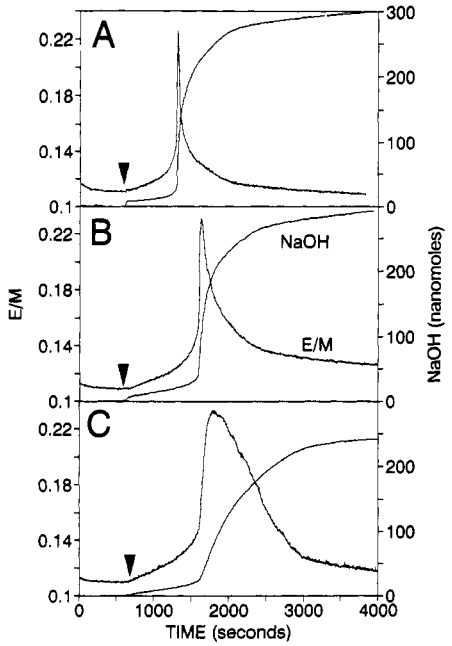


FIGURE 1: Simultaneous measurement of the pyrene excimer: monomer ratio and product accumulation. The arrowheads mark the addition of $PLA_2$. Final [$PLA_2$] was varied: (A) 0.3 $\mu$M; (B) 0.15 $\mu$M; (C) 0.075 $\mu$M. The reaction conditions were 300 $\mu$M DPPC, 0.6 $\mu$M 1-pyrenyldecanoate, 50 mM KCl, and 10 $\mu$M $CaCl_2$ in a total volume of 2.5 mL. The titrant was 2.5 mM NaOH in 50 mM KCl and 10 $\mu$M $CaCl_2$. The temperature was 37.3 (±0.05) °C. The first 600 s of the titration data was used to construct a base line, and the titration data shown have been corrected for the base-line drift.

± 0.05 °C.) As we will show, the phase diagram indicates that these vesicles are entirely in the gel state at this temperature.

The data in Figure 1 allow a detailed examination of the temporal relationship between a marker of lipid disposition ($E/M$) and product accumulation. The $E/M$ increases slowly and substantially prior to the "burst" (the point of most rapid change in enzyme activity) and then very rapidly near the burst. In panel A, the reaction rate is so fast that it is difficult to distinguish the temporal relationship between the maximum in $E/M$ and the burst. At lower reaction rates (panel C), it is clear that the burst precedes the point of maximum $E/M$. While the burst and the $E/M$ maximum are clearly distinguishable, the maximum in $E/M$ may occur simultaneously with the point of most rapid product accumulation.

Gross vesicular structure was not disrupted by hydrolysis. While there are several reports that small unilamellar vesicles (SUV) remain intact during the hydrolysis process (Jain et al., 1986; Bhamidipati & Hamilton, 1989; Wilschutt et al., 1979), we know of no similar reports concerning LUV. Apparently, the entire outer monolayer of SUV can be hydrolyzed without significantly compromising membrane permeability or flip-flop of lipids between the inner and outer monolayers. We infer a similar situation exists using LUV at low concentrations of calcium (5–10 $\mu$M). Concentrations of calcium higher than 100 $\mu$M caused a large increase in light scattering after the burst (Gheriani-Gruszka et al., 1988). The increased light scatter was associated with hydrolysis of all the substrate and seems to be due to the formation of a calcium palmitate precipitate. However, at the low concentrations of calcium used in these studies, there was little change in light scatter, and never more than 50% of the phospholipid was hydrolyzed at the apparent end point of the reaction (Figure 1). The permeability of DPPC LUV during hydrolysis at 10 $\mu$M $CaCl_2$ was assessed by simultaneously monitoring product accumulation and the fluorescence of an internally

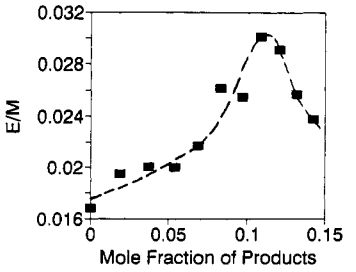


FIGURE 2: Dependence of the pyrene excimer:monomer ratio at 38 °C on the mole fraction of reaction products in LUV made from ternary codispersions containing various amounts of reaction products. The reaction products, monopalmitoyl-PC (lyso-PC) and palmitic acid (PA), are in a 1:1 molar ratio. The mole fraction of reaction product is defined as [lyso-PC]/([DPPC] + [lyso-PC] + [PA]). Conditions: 500 $\mu$M DPPC, 0.5 $\mu$M 1-pyrenyldecanoate, 50 mM KCl, 10 $\mu$M $CaCl_2$, and 10 mM sodium borate at pH 8.0. The values shown here are the initial $E/M$ values for the data in Figure 5.

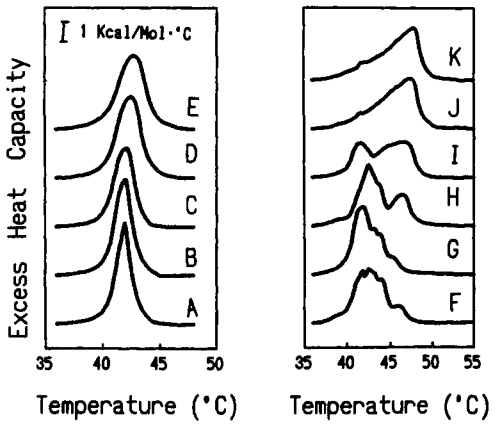


FIGURE 3: Excess heat capacity functions for extruded DPPC large unilamellar vesicles in the presence of various amounts of the reaction products monopalmitoyl-PC and palmitic acid. Monopalmitoyl-PC and palmitic acid are in a 1:1 molar ratio. 5 mM DPPC; buffer: 50 mM KCl, 0.01 mM $CaCl_2$, and 10 mM sodium borate, pH 8.0. Mole fraction of reaction product in each sample: (A) 0.0; (B) 0.019; (C) 0.037; (D) 0.054; (E) 0.069; (F) 0.083; (G) 0.097; (H) 0.109; (I) 0.121; (J) 0.132; (K) 0.143. The mole fraction of reaction product is defined as [lyso-PC]/([DPPC] + [lyso-PC] + [palmitic acid]). The scan rate was 20 C/h. No correction has been made for the time response of the calorimeter.

trapped dye. While leakage of the dye does increase during hydrolysis (and especially near the burst), at least 20% of a dye remains trapped at the apparent end point (data not shown). Also, negative-stain electron microscopy showed that large vesicles remained at the end of a prolonged hydrolysis time course even when the $PLA_2$:vesicle ratio was 100:1 (data not shown).

Figure 2 shows the ratio of excimer to monomer fluorescence intensities ($E/M$) at 38 °C as a function of the mole fraction of products in LUV made from ternary codispersions of DPPC and L-$\alpha$-monopalmitoylphosphatidylcholine (lyso-PC)/palmitic acid (PA) (1:1). The $E/M$ is a maximum at a mole fraction of product between 0.097 and 0.12 (where mole fraction = [lyso-PC]/([DPPC] + [lyso-PC] + [PA]) and [PA] = [lyso-PC]). These data are consistent with the hypothesis that the $E/M$ for 1-pyrenyldecanoic acid is sensitive to some equilibrium, product-dependent property of the membrane such as lateral phase separation of products and substrate.

A partial phase diagram for the ternary mixture of substrate and products (described above) was constructed using differential scanning calorimetry data (Figure 4). The essential features of these data were the same for codispersions which had and had not been extruded (data for multilamellar vesicles are not shown). The heat capacity curves used to generate the phase diagram are shown in Figure 3. The curves were

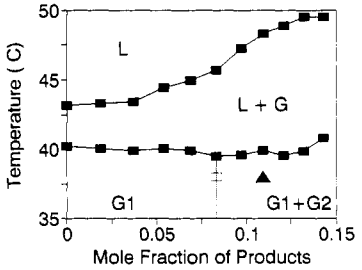


FIGURE 4: Partial phase diagram for the ternary mixture of DPPC, monopalmitoyl-PC, and palmitic acid. Monopalmitoyl-PC and palmitic acid are in a 1:1 molar ratio. The phase diagram is based on the excess heat capacity functions shown in Figure 3. L, liquid-crystalline phase; G1, gel phase enriched in DPPC; G2, putative gel phase enriched in reaction product. The dotted line represents the inferred gel/gel immiscibility boundary. The point on the solidus line is the only point of the immiscibility boundary which can be determined by DSC. This boundary can be represented as a vertical line only if demixing has a small enthalpy and, hence, only a negligible temperature dependence. The open box marks the lowest mole fraction of products which abolished the lag at 38 °C (see Figure 5). The composition which resulted in the largest $E/M$ at 38 °C is denoted by the solid triangle (see Figure 2).

reproducible on repeated heating scans. Comparison of cooling scans with heating scans indicated no hysteresis for curves A through E. However, cooling scans for mole fractions of product greater than 0.083 exhibited hysteresis. The fluidus and solidus lines on the phase diagram were determined from the onset and offset temperatures for the gel to liquid-crystalline transition defined by the heating scans. [Note that reaction products increase the $T_m$ of the pure DPPC. In fact, the 1:1 binary mixture of lyso-PC and palmitic acid forms bilayers and has two transitions, at about 42.5 and 47 °C (Jain et al., 1980).] Within the range of compositions examined, the position of the solidus line with respect to temperature is independent of the mole fraction of products. Above 0.083 mole fraction of products, the excess heat capacity function has two or more maxima. The lack of dependence of the solidus line on the composition and the multiple maxima in the heat capcity together strongly suggest that the mixing of reaction products and substrate is poor in the gel state and that lateral phase separation is likely. Furthermore, the critical mole fraction for phase separation appears to be near 0.083, which correlates well with the mole fraction of product which produced the largest $E/M$ (Figure 2).

The presence of reaction products in the substrate vesicle has been shown to shorten the lag period for hydrolysis. Figure 5 shows $E/M$ values during hydrolysis of LUV composed of ternary mixtures of DPPC, monopalmitoyl-PC, and palmitic acid. With mole fractions of reaction products equal to or greater than 0.083, the lag is absent or too short to observe.

While the phase diagram represents the equilibrium distribution of products and substrate, the $E/M$ recorded during hydrolysis may reflect the kinetics of the putative rearrangements of lipids within the bilayer. Kinetic effects on the $E/M$ were evaluated by rapidly stopping hydrolysis and monitoring subsequent changes in the $E/M$. Because the species of $PLA_2$ used in this study (AppD-49) requires calcium for activity, we attempted to rapidly quench catalysis by adding an excess of the chelating agent EGTA. The efficacy and speed of quenching by EGTA cannot be monitored by the autotitrator, our usual method of measuring product accumulation, because EGTA functions as a buffer at pH 8.0. A suitable alternative for measuring $PLA_2$ activity in a buffered system was needed.

1,2-Bis(pyrenyldecanoyl)phosphatidylcholine is a substrate for $PLA_2$. This molecule gives rise to a large $E/M$, presumably due to intramolecular excimer formation. Hydrolysis at the

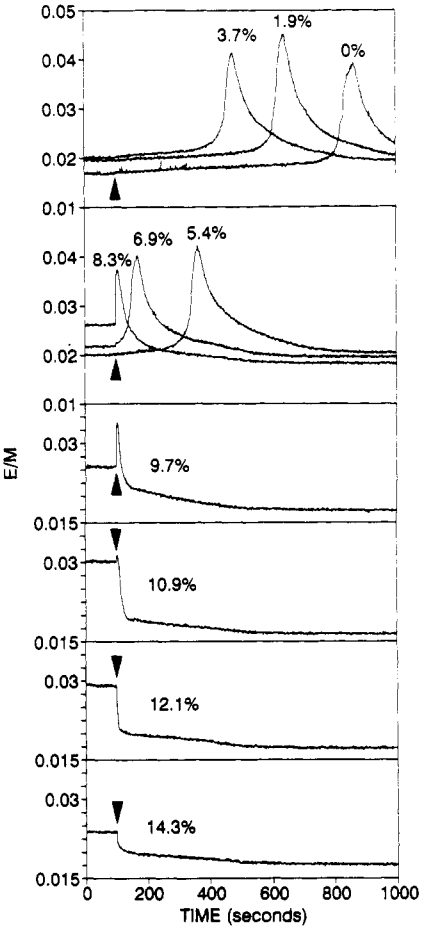


FIGURE 5: Time courses of the excimer:monomer ratio during hydrolysis of LUV made from ternary codispersions containing various amounts of reaction products. The conditions are exactly as described in the legend to Figure 2. $PLA_2$ was added at 100 s (arrows). [$PLA_2$] = 0.3 $\mu$M. The temperature was 38 °C.

ester linkage releases one of the pyrene-labeled fatty acids, and the $E/M$ falls (Hendrickson & Rauk, 1981). Figure 6 (panel A) shows the simultaneous recordings of the $E/M$ and the product accumulation measured by the autotitrator using LUV composed of DPPC/1,2-bis(pyrenyldecanoyl)phosphatidylcholine (1000:1) as substrate. These data show that this bis(pyrene)-labeled phospholipid is a good substrate and is hydrolyzed rapidly at the burst. [In fact, bis(pyrene)-labeled phospholipid seems to be preferentially hydrolyzed. Another curiosity associated with this assay is the observation that all the bis(pyrene)-labeled phospholipid is hydrolyzed.] Therefore, 1,2-bis(pyrenyldecanoyl)phosphatidylcholine was used in a buffered system to determine the approximate rate of inhibition of $PLA_2$ by chelator.

Panel B of Figure 6 shows the effect of the addition of a large excess of EGTA (pH 8.0) to a buffered hydrolysis reaction of LUV composed of DPPC/1,2-bis(pyrenyldecanoyl)phosphatidylcholine (1000:1). A time course of an unquenched reaction is shown for comparison. Note that the addition of chelator abruptly halted the change in $E/M$. The $E/M$ remained stable over the following 25 min. The inset is an expansion of the time points near the addition of EGTA. These data indicate that the reaction can be completely stopped with a mixing time of less than 2 s.

Given the ability to rapidly and completely inhibit hydrolysis, kinetic limitations on the rate of change in $E/M$ can be assessed. The hydrolysis of LUV composed of DPPC/pyrenyldecanoate (500:1) was observed by monitoring the $E/M$, and the reaction was then quenched with EGTA. Figure 7 is a typical example of such a time course. EGTA was

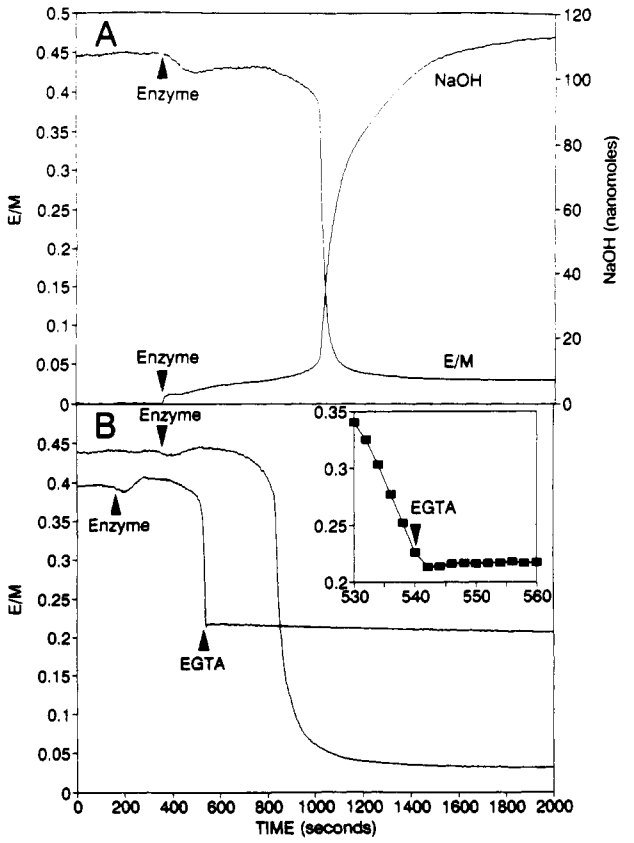


FIGURE 6: Use of 1,2-bis(pyrenyldecanoyl)phosphatidylcholine to monitor the hydrolysis reaction and to approximate the rate of inhibition by chelator. (A) Using DPPC/1,2-bis(pyrenyldecanoyl)-phosphatidylcholine (1000:1) and the autotitrator, the pyrene excimer: monomer ratio and product accumulation were measured simultaneously. 200 nM $PLA_2$ was added to the cuvette at the arrowheads. The reaction conditions were 300 $\mu$M DPPC, 0.3 $\mu$M 1,2-bis-(pyrenyldecanoyl)phosphatidylcholine, 50 mM KCl, and 10 $\mu$M $CaCl_2$, in 2.5 mL. The titrant was 2.5 mM NaOH in 50 mM KCl and 10 $\mu$M $CaCl_2$. The temperature was 37.3 (±0.05) °C. Titration data prior to the addition of $PLA_2$ were used to construct a base line. The titration data shown have been corrected for the base-line drift. (B) Using DPPC/1,2-bis(pyrenyldecanoyl)phosphatidylcholine (1000:1), the rate of inhibition by chelator was approximated. In each of two experiments, 400 nM $PLA_2$ was added to the cuvette at the arrowhead. In one experiment, 32 $\mu$L of 500 mM EGTA (pH 8.0) was added at the marked arrowhead. The inset is a detail of the region of addition of EGTA. Each data point is an average over 1 s, and the data points are 1 s apart. The reaction conditions were 300 $\mu$M DPPC, 0.3 $\mu$M 1,2-bis(pyrenyldecanoylphosphatidylcholine, 10 mM HEPES (pH 8.0), 50 mM KCl, and 10 $\mu$M $CaCl_2$, in 1.6 mL.

added during the rapid increase in $E/M$. The $E/M$ continued to change rapidly for several seconds, well after the 2-s mixing time. Also, the $E/M$ drifted toward an apparent equilibrium value for minutes after the reaction was quenched. The addition of EGTA did not change the pH of the reaction (data not shown). Injection of an equal volume of 500 mM KCl also had no effect on the time course, indicating that osmotic shock cannot account for the effect of EGTA (data not shown). Therefore, the $E/M$ recorded during the time course of hydrolysis does not reflect exactly an equilibrium disposition of pyrenyldecanoate in the membrane.

## DISCUSSION

*Equilibrium Lateral Phase Separation in a Ternary Mixture of DPPC and Lyso-PC/Palmitic Acid and Activation of Phospholipase $A_2$.* The data presented here support the proposal that lateral phase separation due to gel/gel immiscibility is related to the dramatic increase in the activity of $PLA_2$ at temperatures below the main phase transition. The partial phase diagram for the ternary mixture of substrate

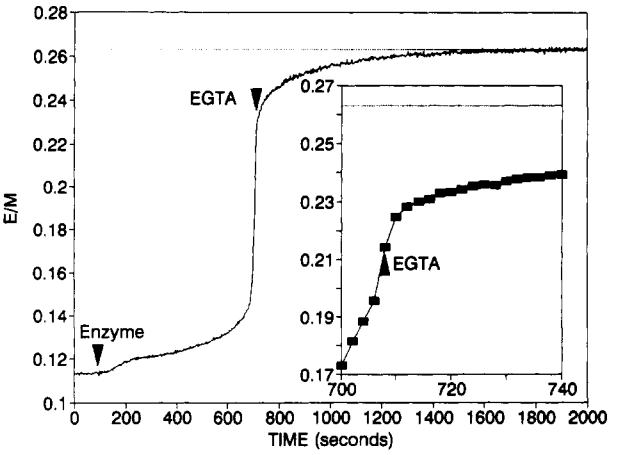


FIGURE 7: Effect of rapidly stopping hydrolysis on the time course of the change in $E/M$. 200 nM $PLA_2$ was added to the cuvette at the arrowhead. The arrowhead at 708 s marks the addition of 32 $\mu$L of 500 mM EGTA. The inset is a detail of the region of addition of EGTA. The horizontal dotted line is the average of the $E/M$ ratio measured between 1940 and 2000 s. The reaction conditions were 300 $\mu$M DPPC, 0.6 $\mu$M 1-pyrenyldecanoate, 10 mM HEPES (pH 8.0), 50 mM KCl, and 10 $\mu$M $CaCl_2$ in 1.6 mL. The temperature was 37.55 (±0.05) °C.

and reaction products indicates that the critical mole fraction of product for demixing at 39.5 °C (the solidus line) is between 0.069 and 0.083. The heat capacities for the ternary mixtures were reproducible on repeated heating scans, indicating that the coexistence of compositionally distinct phases in the gel state is an equilibrium phenomenon. Jain and de Haas (1981) report that at 0.23 mol fraction of reaction products in DPPC, there is demixing as demonstrated by DSC. No data were reported for lower mole fractions of products, those concentrations which are relevant to the rapid activation process of $PLA_2$ (i.e., the burst).

The $E/M$ as function of mole fraction of products at 38 °C passes through a maximum between 0.097 and 0.12 mol fraction. This maximum in $E/M$ as a function of reaction products in ternary codispersions is observed near the putative critical demixing concentration identified by DSC at 39.5 °C. Thus, the maximum $E/M$ may be thought of as a marker for demixing.

The absolute value of $E/M$ in the ternary mixtures prior to hydrolysis is markedly less then that observed during hydrolysis (Figure 5). The many possible reasons for this difference include (1) extreme sensitivity of $E/M$ to the mole fraction of product, (2) nonequilibrium distributions of probe and phospholipid during hydrolysis, and (3) the difference between distributing product in both leaves of the bilayer (by codispersion) and only one leaf (by hydrolysis of the outer monolayer). Regardless of this difference in absolute magnitudes of $E/M$, the existence of a maximum as a function of product concentration strongly indicates that this observable is sensitive to a structural transition in the bilayer.

There is striking correlation between the product dependence of lateral phase separation as identified by DSC (and corroborated by the maximum in the $E/M$) and the product dependence of rapid activation of $PLA_2$. The mole fraction of product present at the end of the lag using DMPC SUV at 17 °C is approximately 0.05 (Apitz-Castro et al., 1982), and the amount of product necessary for abolition of the lag using LUV of ternary mixtures of DPPC and lyso-PC/PA (1:1) is between 0.069 and 0.083 mol fraction (Figure 5). It is thus clear that the length of the lag phase is a reasonably accurate measure of the time required to produce a constant mole fraction of product, an assumption which has been used

to analyze the substrate dependence of $PLA_2$ activation (Bell & Biltonen, 1992).

*Dynamic Lateral Phase Separation of Substrate and Product and Activation of Phospholipase $A_2$.* If lateral phase separation is essential for the rapid activation of $PLA_2$, then lateral rearrangement of lipid must precede the burst. It is apparent from the simultaneous recordings of $E/M$ and product accumulation that the environment of the 1-pyrenyldecanoate changes slowly but substantially during the lag period and then changes abruptly at the burst. Therefore, lateral rearrangement of lipids on an appropriate time scale for coupling with activation is likely.

The simplest model that we imagine for $E/M$ variation with product concentration is that 1-pyrenyldecanoate preferentially partitions into regions enriched with product. The first product-enriched regions will contain a disproportionate amount of pyrene-labeled fatty acid. These regions of relatively aggregated 1-pyrenyldecanoate will give rise to a higher $E/M$ than the initial homogeneous mixture of probe in DPPC. As these product-enriched domains grow, the 1-pyrenyldecanoate will be diluted and the $E/M$ will drop, eventually returning to its original value once the entire monolayer has been hydrolyzed.

The excimer:monomer ratio is determined by the concentration of pyrene and by its diffusion coefficient; increasing either results in an increase in the $E/M$ (Birks, 1963). In this system, the $E/M$ data show that either the lateral distribution of 1-pyrenyldecanoate changes or its lateral mobility changes, or both. A quantitative analysis of the relationship between lateral phase separation and the $E/M$ requires detailed models for both lateral phase separation and excimer formation. Models for these processes remain controversial. The most recently published models of excimer formation in two-dimensional, viscous solutions add substantial complexity to the original model of Birks (Sugar et al., 1991). Also, experimental work indicates that certain fundamental assumptions used in quantitative modeling about the distribution of pyrene-labeled phospholipids may be wrong (Somerharju et al., 1985). However, some qualitative understanding of the relationship between observed excimer formation and phase separation is possible and informative.

On the basis of our observations, the most likely source of variation in $E/M$ is the change in local concentrations of 1-pyrenyldecanoate (or a change in a local diffusion coefficient) due to lateral phase separation, and not a change in bulk diffusion rates unaccompanied by phase separation. Two observations lead to this conclusion. First, nonequilibrium distribution of pyrenyldecanoate implies that lateral redistribution of lipid is involved in the change in $E/M$. If only changes in the average diffusion coefficient of the pyrene-labeled fatty acid occurred without a redistribution, then one would expect that the changes in $E/M$ would cease upon termination of the reaction. Our data show that the hydrolysis reaction stops within 2 s following addition of EGTA while the change in $E/M$ persists for hundreds of seconds. Second, excimer fluorescence, monomer fluorescence, and, therefore, the $E/M$ are each nearly the same at the beginning and end of the hydrolysis reaction. Assuming that at the reaction end point the entire outer monolayer is hydrolyzed and no lateral phase separation still exists, then the total area accessible to the probe at the start and end of the reaction is approximately the same. Therefore, the probe resides in a single phase, gel state at both the beginning and the end of the reaction. These facts imply that pyrenyldecanoate has the same diffusion coefficient in gel-state DPPC and gel-state 1:1 lyso-PC/palmitic acid. Between these two extremes is a region of two-phase coexistence and a maximum in the $E/M$. Regardless of apparent similarity of diffusion coefficients in the pure substrate and pure product domains, this maximum in $E/M$ might result from increased diffusion in the mixed-gel region, perhaps within some region of defective packing at boundaries of domains. However, again, this scenario necessarily implies that lateral phase separation has occurred.

*Temporal Correlation of Lateral Phase Separation and Activation.* If a certain membrane structure effects activation of $PLA_2$, then that structure must be assumed prior to the rapid change in activity. Bell and Biltonen (1989) reported a sudden change in the fluorescence of a membrane probe (trimethylammoniodiphenylhexatriene) which occurs just prior to the burst. The simultaneous data here clearly show that the $E/M$ changes substantially before the burst occurs. Using the arguments above concerning the origin of the change in the $E/M$, these early events are ascribable to the formation of small domains enriched in products.

Our data agree generally with a report of parallel measurements of product accumulation and an optical marker presumed to reflect lateral phase separation (Jain et al., 1989). However, the data presented here include simultaneous observations of product accumulation and a marker of lateral phase separation. Inherent in a system which displays critical sensitivities is a substantial degree of variation between apparently identical samples. Therefore, the simultaneous data acquisition allows some comments on the details of the relationship between lateral phase separation and activation of $PLA_2$.

The nonequilibrium distribution of pyrenyldecanoate during the rapid phase of change in the $E/M$ shows that $PLA_2$ changes the composition of the membrane far faster than lateral rearrangement of lipid can achieve equilibrium in the phase-separated membrane. This situation raises the possibility that dynamic local conditions may affect $PLA_2$ activity. For example, the rate of hydrolysis may be limited by product inhibition which can only be relieved by diffusion of product away from the active enzyme (Lathrop & Biltonen, 1992).

*Theories Concerning the Role of Membrane Structure in the Critical Activation Process of Phospholipase $A_2$.* Beginning with Op den Kamp et al. (1974), defect formation in the membrane surface has been repeatedly invoked to rationalize the apparently critical activation process. Various manipulations of zwitterionic membranes which are presumed to produce surface inhomogeneities have been shown to alter the lag time (Tinker et al., 1978; Wilschutt et al., 1979; Menashe et al., 1986; Lichtenberg et al., 1986; Sen et al., 1991). Spurred by the observation of a lag time, a role for reaction products in the activation of $PLA_2$ was proposed by Wilschut et al. (1979) and Tinker et al. (1978). Early studies by Dawson (1958a) on PLA B showed that that the enzyme's activity toward lecithin is extremely sensitive to a critical mole fraction of an "activating lipid". Apitz-Castro et al. (1982) noted that there appears to be a fixed mole fraction of reaction products at the time of the burst in $PLA_2$ activity. These data lead to the hypothesis that lateral phase separation shows a critical dependence on the mole fraction of product and that lateral phase separation is necessarily accompanied by defect formation.

Grainger et al. (1989, 1990) have studied $PLA_2$ activity in the gel/liquid-crystalline coexistence region. These elegant studies used a liquid phase soluble fluorescent probe in zwitterionic monolayers to visualize the location of $PLA_2$ activity at the gel/liquid boundary region. Their striking images make it clear that $PLA_2$-catalyzed hydrolysis perturbs the gel/liquid boundary region and that this perturbation

originates at specific defects within the boundary region. However, these studies did not include measurements of product accumulation. Therefore, while these studies localized the sorts of perturbations which $PLA_2$ activity can cause, the effect of membrane structure on activity was not directly addressed.

Another activation mechanism was suggested by the behavior of $PLA_2$ toward vesicles composed of anionic lipids (Jain & Berg, 1989). No lag is observed when certain negatively charged vesicles are used as a substrate. Early work on PLA B suggested that anionic lipids were particularly effective as "activating lipids" (Dawson, 1958b). Dawson also suggested (1969) that the electrostatic field at the membrane/water interface could dramatically affect $PLA_2$-catalyzed hydrolysis. Noting that one reaction product is anionic, the fatty acid, Jain et al. (1989) rationalized the activity's dependence on reaction products on electrostatic grounds and differences in the apparent affinity of $PLA_2$ for charged and neutral membranes. Implicit in this model is a requirement for a critical charge density, a model previously developed to explain PLA B activity (Bangham & Dawson, 1959). Such a charged density could be achieved by aggregating the charged species, an event linked to the lateral phase separation of reaction products. Jain et al. (1989) suggested such a mechanism for the activation of $PLA_2$. However, this model implies that the region to which $PLA_2$ binds is very poor in substrate, a situation unlikely to cause a large increase in the apparent activity. Also, the zwitterionic product, lyso-PC (Jain & de Haas, 1983; Bell & Biltonen, 1992), and neutral diacylglycerols (Bell et al., 1991) can alone shorten or abolish the lag time. Fatty acid alone has been reported to have no effect or even prolong the lag time (Bell & Biltonen, 1992). Therefore, while lateral phase separation is related to the critical activation phenomena, the actual molecular role of these membrane structures in activation is still mysterious.

The apparent central role of lateral phase separation in the rapid activation process may help to rationalize the nonlinear relationship between enzyme concentration and lag time (Romero et al., 1987). A number of authors have postulated that the active form of the enzyme is a dimer (Tomasselli et al., 1989; Roberts et al., 1977; Cho et al., 1988; Romero et al., 1987; Bell & Biltonen, 1992). This enzyme is a monomer in solution and is presumed to dimerize on a vesicle's surface. The rate or extent of dimer formation could be enhanced if the local monomer concentration were increased by preferential association with specific domains on the membrane (Thompson et al., 1992). Whether this is true remains to be demonstrated.

Lateral phase separation appears to be a mechanism by which membrane structure can modify the behavior of a membrane-associated enzyme. Here, we demonstrate the existence of lateral phase separation even at the low mole fractions of products reported at the burst in $PLA_2$ activity. This phase separation is rapid enough to allow it to play a role in $PLA_2$ activation on gel-state membranes. These observations may explain not only the critical product concentration effect on $PLA_2$ activity but also the ability of some small hydrophobic molecules to modulate $PLA_2$ activity.

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