Direct Visualization of the Dynamic Hydrolysis Process of an L-DPPC Monolayer Catalyzed by Phospholipase D at the Air/Water Interface

Qiang He, Xiuhong Zhai, and Junbai Li*

International Joint Lab, Key Laboratory of Colloid and Interface Science, The Center for Molecular Sciences, Institute of Chemistry, Chinese Academy of Sciences, Zhong Guan Cun, Beijing 100080, China

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The hydrolysis reaction of L-α-dipalmitoylphosphatidylcholine (L-DPPC) catalyzed by cabbage phospholipase D (PLD) at the air/water interface has been studied by Brewster angle microscopy (BAM) and film balance technique. The curves of surface pressure versus time show that the hydrolysis reaction depends on the initial state of the lipid monolayer. The BAM images display that the hydrolysis reaction preferentially occurs in the liquid-expanded phase where PLD has a maximum activity. In the coexistence region of liquid-expanded and liquid-condensed phases, a "lotus-like" domain of the monolayer was observed, indicating that the hydrolysis product 1, 2-dipalmitoylphosphatidic acid (DPPA) created a new phase, which inhibited the reaction rate of hydrolysis.

Introduction

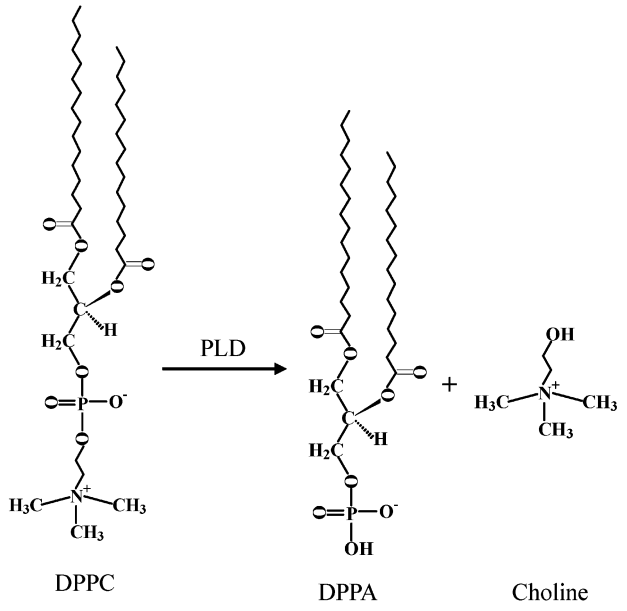
All chemical reactions in living cells depend on enzyme catalysis. Enzyme activities are frequently dependent on their mechanism of catalysis. However, the mechanisms by which they are activated are largely unknown.

One important enzyme is phospholipase D (PLD) (E.C.3.1.4.4), that catalyzes the cleavage of the terminal phosphate ester bond at the polar headgroup of phosphatidylcholine to phosphatidic acid and a water-soluble choline (Scheme 1).[1] This enzyme is widely present in plants, bacteria, and animals and is involved in membrane formation, protein transport, regulation of mitosis, and so on.[2] Another interest in the action of PLD also derives from one of the PLD cleavage products, phosphatidic acid, a second messenger that plays an important role in signal transduction pathways.[3,4] Moreover, phosphatidic acid may be an important source of arachidonic acid for synthesis of prostaglandins and leukotrienes.[5] Therefore, a detailed investigation of the lipid monolayer hydrolysis by PLD in detail is of great significance in the biophysical point of view.

It is known that PLD is a calcium-dependent enzyme acting preferentially on substrate aggregates, such as membranes, vesicles, micelles, or monolayers. PLD activity strongly depends on the physical chemical structure of the substrate.[6] The enzymatic reaction with the membrane surface consists of a molecular recognition process and a cleavage reaction.[7−9]

A suitable approach to aid the understanding of molecular interactions in biological membranes has been established by using the monomolecular films as biomimetic systems.[10−15] This approach offers an opportunity to investigate the dependence of 2-D phase behavior on molecular packing and interactions in a single membrane leaflet. It would also allow us determine the effect of molecular packing on the penetration of the enzyme into the lipid layers during the hydrolysis process. This method only needs a very small amount of phospholipid and can conveniently vary the physicochemical parameters of the reaction, such as temperature, lateral pressure, molecular density, ionic condition, etc. The monomolecular layer is therefore a suitable model for investigating enzyme reactions at the interface.

SCHEME 1: Schematic Representation of the Hydrolysis of an L-DPPC Monolayer by Phospholipase D (PLD)



Based on this approach, many different techniques have been used to investigate the hydrolytic mechanism of phospholipid monolayer by phospholipases, such as two-step measurement,[16] surface X-ray diffraction,[17] IR spectroscopy,[18] fluorescence microscopy,[19] atomic force microscope (AFM),[20] and polarized-modulated infrared reflection adsorption spectroscopy.[9] With these methods, many physical parameters such as thermal and kinetic constants can be readily determined. Recently, Brewster angle microscopy has been considered as a suitable technique to monitor hydrolytic process of lipid monolayers.[21−23]

BAM is based on the physical fact that p-polarized light is not reflected from an interface between two materials with

* To whom correspondence may be addressed. Phone: + 86-10-82614087. Fax: +86-10-82612484. E-mail: jbli@iccas.ac.cn.

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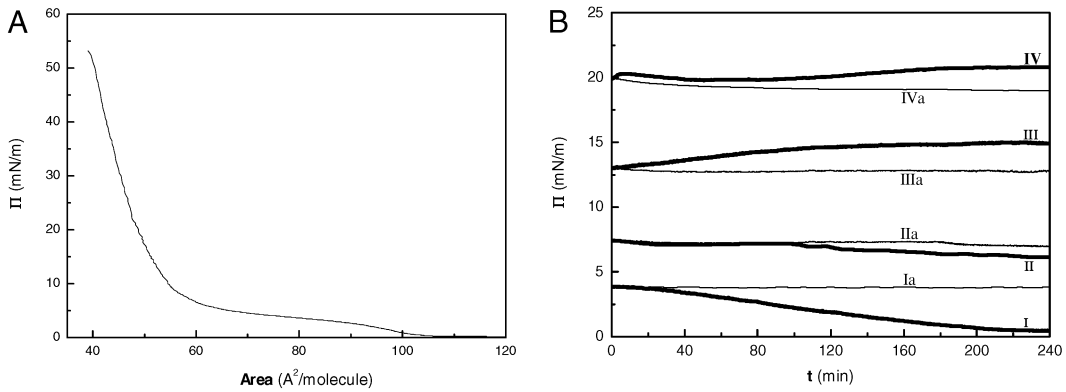

Figure 1. (A) Pressure−area isotherm of L-DPPC monolayer in pure water as a subphase at the air−water interface. (B) Variation of the surface pressure π as a function of time t as PLD was injected into the subphase of the L-DPPC monolayer at different initial pressures: (I) 3.8, (II) 7.5, (III) 13, and (IV) 20 mN/m, respectively. The dotted lines, Ia−IVa, represent the $\pi-t$ curves of L-DPPC monolayer without adding PLD in the subphase corresponding to the initial surface pressures, respectively.

different refractive coefficients if the incident angle is at the Brewster angle. The existence of domains in a Langmuir monolayer changes the local refractive index such that at the Brewster angle for the pure air−water interface, light is now reflected with varying intensity from the different phases. The reflected light can then be used for imaging purposes. BAM can thus be used to visualize details of the inner structure of condensed phase domains at the air/water interface and to follow morphological changes of the reactive process occurring in monolayers under constant surface pressures.[14,15,24]

In the present work, we have combined the monolayer technique with BAM to investigate the hydrolytic reaction of L-DPPC monolayer catalyzed by PLD. The morphological changes of condensed phase domains in enzymatic hydrolytic processes have been directly observed at the air/water interface. A new type of "lotus-like" domain has been observed, which is due to the formation of the insoluble hydrolysis product. The hydrolysis kinetics studied by detecting the surface pressure change with reaction time provides information for the hydrolysis efficiency.

Experimental Section

Phospholipase D (from cabbage, E.C.3.1.4.4) was purchased from ICN Biomedicals Inc. and used without further purification. L-α-Dipalmitoyl-*sn*-glycero-3-phosphatidylcholine (L-DPPC) and L-α-dipalmitoyl-*sn*-glycero-3-phosphatidic acid (DPPA) was received from Sigma. Chloroform (99%) was obtained from ACROS. Analytical-grade sodium acetate, acetic acid, and calcium chloride were used for the subphase. The phospholipids were dissolved in chloroform to prepare 1 mM solution. The subphase was aqueous buffer solution with 40 mM $CaCl_2$, 80 mM CH_3COONa at pH 5.6.

Water was purified with a Millipore desktop system and reached a specific resistance above 18.2 MΩ·cm. The enzyme was dissolved in the same buffer solution as the one used for the subphase. In all experiments, the temperature of the subphase solution was controlled at 20 ± 0.1 °C by circulating water using a RC LAUDA unit. The concentration of the PLD in the subphase was 0.125 U/mL.

The experimental setup used for the measurement at the air/water interface is the same as described previously.[14] A commercial Brewster angle microscope (Optrel, Germany) was mounted onto a computer-controlled Langmuir trough. The reflected light was detected by an analyzer and a CCD camera; the output signal was recorded on a video recorder. The images were captured afterward with a frame grabber and processed using special software to adjust the contrast and correct the image distortion caused by observation done at the Brewster angle. The surface pressure was measured by the Whilhemy method using a small filter paper within ±0.1 mN m^{-1}. The lateral resolution of BAM was about 1 μm.

The Langmuir monolayer was prepared by spreading 30 μL of 1 mM L-DPPC solution on the surface of the subphase by a microsyringe. After about 10 min the monolayer was compressed at a velocity of 2.5 $Å^2$ $molecules^{-1}$ min^{-1} to a desired initial surface pressure. Then, the enzyme solution was injected into the subphase and carefully stirred underneath the monolayer. At the same time, the curve of surface pressure versus time was recorded and the morphological changes of the domains during the hydrolysis process were continually monitored by BAM. It should be noted that once the desired initial surface pressure was reached, the trough area remained constant throughout the entire hydrolysis process.

Mixed L-DPPC/DPPA monolayers were prepared using the same procedure as in the L-DPPC.

Results and Discussion

Figure 1A shows the pressure−area isotherm of a pure L-DPPC monolayer in a pure water subphase at 20 °C. A main phase transition between the liquid-expanded (LE) and liquid-condensed (LC) is observed at 4.5 mN/m. The plateau of the isotherm corresponds to the coexistence of LE and LC phases. The plots of surface pressure as a function of time in the presence (solid lines) and absence (dotted lines) of PLD at different initial surface pressure are shown in Figure 1B. It is seen that the initial surface pressures have an obvious effect on the hydrolysis reaction. When the initial surface pressure is at $\pi_0 = 3.8$ (curve I of Figure 1B) or 7.5 mN/m (curve II of Figure 1B), respectively, where the liquid-expanded phase of the lipid monolayer is enriched, the surface pressure slightly decreases with the reaction time after the injection of the enzyme when compared to the curves Ia and IIa when the enzyme is absent. This demonstrates that the L-DPPC monolayer hydrolysis by PLD takes place at such low surface pressures.

One of the hydrolytic products, choline, is fully water-soluble and can leave the interface into the subphase. The other hydrolytic product, DPPA, has a smaller headgroup and occupies a smaller molecular area than L-DPPC at the same surface pressure. As a consequence, the molecular density at the interface was decreased. Correspondingly, it leads to the reduction of surface pressure. At these low initial surface pressures, the negative slopes of the $\pi-t$ curves I and II in Figure 1B

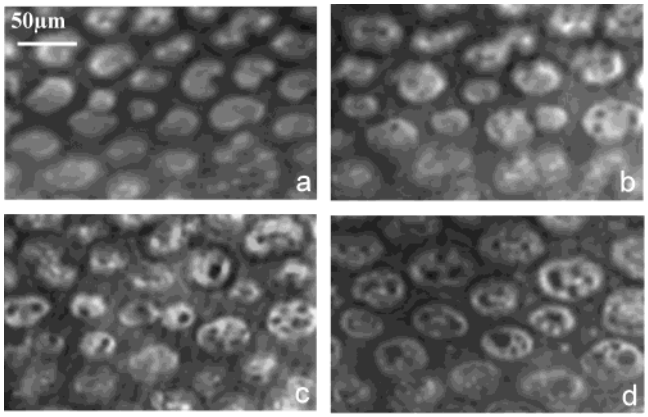

Figure 2. BAM images of an L-DPPC monolayer at the air−water interface recorded with the reaction time during hydrolysis by PLD at an initial surface pressure, $p_0 = 7.5$ mN/m, $C_{PLD} = 20$ U. Domains observed (a) from pure L-DPPC monolayer in the coexistence region, (b) 15 min after the injection of PLC into the subphase, (c) 40 min after hydrolysis reaction, and (d) 120 min after hydrolysis reaction.

demonstrate that the hydrolysis rate is faster than the adsorption rate of enzyme into the lipid monolayer. When the initial surface pressure was increased to $\pi_0 = 13$ and 20 mN/m (curves III and IV, respectively, in Figure 1B), positive slopes in the curves are observed when the enzyme is present as opposed to the flat curves of IIIa and IVa, indicating that there is less hydrolysis reaction. The increase in the surface pressure is mainly ascribed to the adsorption of PLD underneath or partly penetrating into the L-DPPC monolayer. With these surface pressures the lipid monolayer remains in a physical state of LC-enriched or full LC phase. Thus the densely packed film restricts the attack of the enzyme to the lipid. From Figure 1B we can learn that at $\pi = 3.8$ mN/m where the monolayer is in a LE phase there exists the largest surface pressure difference, $\Delta\pi = 3$ mN/m, indicating the highest level of PLD activity. This is obviously different from that of phospholipase A_2 (PLA_2), which hydrolyzes the sn-2 ester linkage of L-DPPC and has maximum activity in the coexistence region of LC and LE phases for monolayer.[14,25] These findings suggest that the aliphatic chain orientation of the substrate molecules is important for the monolayer hydrolysis catalyzed by PLA_2. In contrast, PLD acts in the headgroup of the phospholipid and the highest level of PLD activity is observed when the film is disordered. This indicates that fluidity and defects, as opposed to adsorption-induced orientation of the substrate, in the monolayer may play a key role. These deductions are in good agreement with the results of polarization-modulated infrared reflection absorption spectroscopy (PM-IRRAS).[9]

To prove that this is indeed the case, we have performed BAM measurements and detected the morphological variations of L-DPPC domains during the hydrolysis process at two-phase coexistence region.

BAM images of L-DPPC monolayer at the air−water interface before the injection of PLD into the subphase and after the hydrolysis reaction at $\pi_0 = 7.5$ mN/m are shown in Figure 2. The formed domain (bright) during compressing the L-DPPC monolayer is a LC phase, which means that LC domains in a LE background (dark) (Figure 2a). An obvious morphology change of L-DPPC domains was observed in 15 min after the injection of PLD (Figure 2b). Within the structure of L-DPPC white domains appear some small dark dots, and they are hydrolyzed by PLD into a "lotus" or "Swiss cheese" structure in 40 min (Figure 2c). From the BAM principle, we learned that the same light refractive properties of LC region were caused by the identical molecular orientation in the inner L-DPPC domains.[22] This means that the appearance of the "lotus" domains suggests the production of a new phase in the L-DPPC condensed domain. DPPA should be regarded as a main contributor to this new phase.

The dark dots were gradually enlarged with the increasing reaction time up to 120 min (Figure 2d). Then the mixed "lotus-like" and "O" types of domains remain unchanged for a longer time. It should be noted that L-DPPC LC domains are not completely damaged throughout the entire hydrolysis process. This obviously demonstrates that the product DPPA greatly inhibits the hydrolysis efficiency. On the other hand, the formation of "lotus-like" and "O" types of domains in the hydrolysis process means that the hydrolysis reaction of phospholipid monolayer catalyzed by PLD also occurred in the ordered phase but its hydrolysis reaction was not completed. Maybe it also happens in the disordered phase but there is not enough change in the resulting refractive index to see it.

At a higher initial surface pressure, $p_0 = 13$ mN/m, L-DPPC monolayer is completely in the liquid condensed phase. It is found that no domains or film defects are observed after PLD injection into the subphase. This is consistent with the result obtained from the isotherm (Figure 1B, curve IIIa). We have to point out here that although the domains observed in the two-phase coexistence regions are contributed originally from the LC phase, whereas no domains are observed in the pure LC phase. This is because that the hydrolysis rate in the LC phase is extremely slow comparing to that in the coexistence region, which thus will cause a small change of the refractive index so that we are unable to observe the possible hydrolysis process.

To explore the "lotus" domain structure in details, we have compared the domain features of mixed L-DPPC/DPPA monolayers. Figure 3A shows the $\pi-A$ isotherms of mixed L-DPPC/DPPA monolayer in a buffer solution as a subphase with different ratios at 20 °C. The isotherm shifts toward the right with increasing mole ratio of L-DPPC and has a larger slope at a higher surface pressure. It shows that the decreasing DMPA content in the DPPC monolayer increased the apparent molecular area.

When the film is pure L-DPPC (curve f in Figure 3A), the isotherm displays a plateau region, signifying the coexistence of LC and LE phases. Meanwhile we deduce that DPPA and L-DPPC have a weak compatibility. The BAM micrographs in Figure 3B show that the increasing DPPA mole fraction from 10% to 40% at the same initial surface pressure below the plateau region results in the disappearance of the liquid condensed domains. The addition of DPPA may significantly enlarge the nucleation numbers of L-DPPC molecular aggregations. As a consequence DPPA inhibits the formation of large L-DPPC domains. This can be proved by the complete disappearance of the LC domains, while the mole fraction of DPPA increases from 10% to 40%. This furthermore demonstrates that phase separation exists in mixtures of L-DPPC and DPPA in monolayers when DPPA has a low molar fraction. Meanwhile, the hydrolysis product DPPA will be enriched in the L-DPPC monolayer, leading to an inhibition of the ongoing enzymatic reaction of PLD. Grazing incidence X-ray diffraction (GIXD) investigation of a similar mixture system also verifies that the headgroup conformation of DPPA remains unchanged in the mixture.[26]

On the basis of the above experiments, we have learned that the phospholipid monolayer, when hydrolyzed by PLD at the interface becomes a binary mixture of the hydrolytic product, DPPA, and the substrate L-DPPC. The "lotus" domains observed

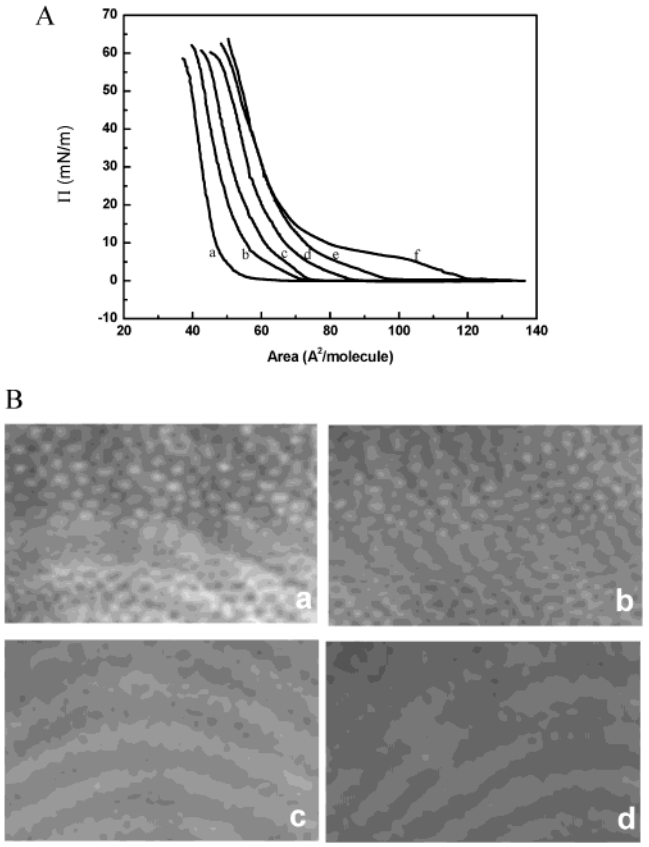

Figure 3. (A) Surface pressure−area isotherm of mixed L-DPPC and DPPA monolayer on the buffer with 40 mM $CaCl_2$, 80 mM CH_3COONa at pH 5.6 in a different molar ratio. DPPA molar ratios in DPPC monolayer were (a) 100%; (b) 90%; (c) 50%; (d) 40%; (e) 20%; (f) 0%. (B) BAM images of mixed L-DPPC/DPPA monolayer at the same surface pressure below plateau regions. (a) 10% DPPA; (b) 20% DPPA; (c) 40% DPPA; (d) 50% DPPA.

in the hydrolyzed monolayer resulted from the disordered DPPA (dark) and ordered L-DPPC domains (bright).

In the PLA_2 hydrolysis experiment, there are new types of brighter domains formed at a higher surface pressure, for instance, when $\pi = 15$ mN/m.[14] Fluorescence microscopy studies[27] have confirmed that those are PLA_2 domains. However, in the present study at equivalent initial surface pressure no obvious white and huge domains are observed. This indicates that no PLD domains penetrating into the lipid monolayer are present. The hydrolysis process of L-DPPC by PLD can be carried out underneath the lipid monolayer.

From the observation above, it can be seen that the L-DPPC monolayer hydrolysis by PLD started at the weak or defect points of the ordered domains. Further hydrolysis of L-DPPC monolayer creates a locally higher concentration of hydrolytic product DPPA in the monolayer. This may initially activate the process of the hydrolysis reaction. This activated action could be initiated by Ca^{2+} ions that induce deprotonation of the DPPA headgroup and form a Ca^{2+}−DPPA complex.[26,28−30] The interaction may promote the interfacial binding of PLD to the lipid monolayer and lead to the increase of hydrolysis rate. However, once the hydrolysis reaction carries through, the incompatibility of the hydrolytic product DPPA and the nonreactant L-DPPC will inhibit the hydrolysis reaction of lipid monolayer and finally result in the incomplete cleavage of the L-DPPC monolayer. This observation is in good agreement with those previously reported.[27,31,32] In conclusion, the phase separation of the hydrolytic product and the substrates in one monolayer may play a key role in initially activating the hydrolysis process and then inhibiting the hydrolytic reaction.

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