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Dynamic Observations of the Hydrolysis of a DPPC Monolayer at the Air/Water Interface Catalyzed by Phospholipase A_2**

Junbai Li,* Zhijian Chen, Xiaoli Wang, Gerald Brezesinski, and Helmuth Möhwald*

Many chemical reactions, especially those in the crystalline state, depend on the local environment of the reactants. Hence in order to study their mechanism one has to investigate these reactions as a function of local order, and in addition, the change of order should be measured as the reaction proceeds. For this purpose amphiphile monolayers are highly suited, because their order can be varied and characterized, and the chemical reaction can be followed by newly developed spectroscopic and microscopic techniques. In addition the reactants can be exchanged with the adjacent phase in a controlled way.

One biologically and technologically important system is the phospholipase A_2 (PLA_2) that catalyzes the hydrolysis of a phospholipid monolayer at an interface. It is known that this reaction depends upon the state of the monolayer. It is now possible to study the molecular arrangement within monolayers, even in individual domains, with microscopic resolution. For this investigation the main methods used are surface X-ray diffraction, FT-IR spectroscopy, and Brewster angle microscopy (BAM). These techniques are used here to follow the enzyme reaction in detail. The correlation of changes in the molecular arrangement with catalytic activity should provide an understanding of reaction mechanisms.

PLA_2 is a calcium-dependent enzyme abundant in living organisms. The enzymatic reaction with the membrane surface consists of a molecular recognition process and a cleavage reaction.[1] PLA_2 stereoselectively hydrolyzes the *sn*-2 ester linkage of L-phospholipids to release fatty acids and lysophospholipids (Figure 1a). The activity of PLA_2 at the interface of aggregated substrates, such as phospholipid monolayers, is 10000-fold greater than with the corresponding monomeric substrate.[2] PLA_2 has an α-helix-enriched conformation in aqueous solution (Figure 1b). During an enzymatic reaction a change in this conformation may occur as soon as the enzyme reaches the interface.

[*] Prof. Dr. J. Li, Z. Chen, X. Wang
International Joint Lab, Center for Molecular Science
Institute of Chemistry, Chinese Academy of Science
De Wai, Bei Sha Tan, Beijing 100101 (China)
Fax: (+86) 10-6487-9375
E-mail: jbli@ipc.ac.cn

Prof. Dr. H. Möhwald, Dr. G. Brezesinski
Max Planck Institute of Colloids and Interfaces
14476 Golm/Potsdam (Germany)
Fax: (+49) 331-567-9202
E-mail: moehwald@mpikg-golm.mpg.de

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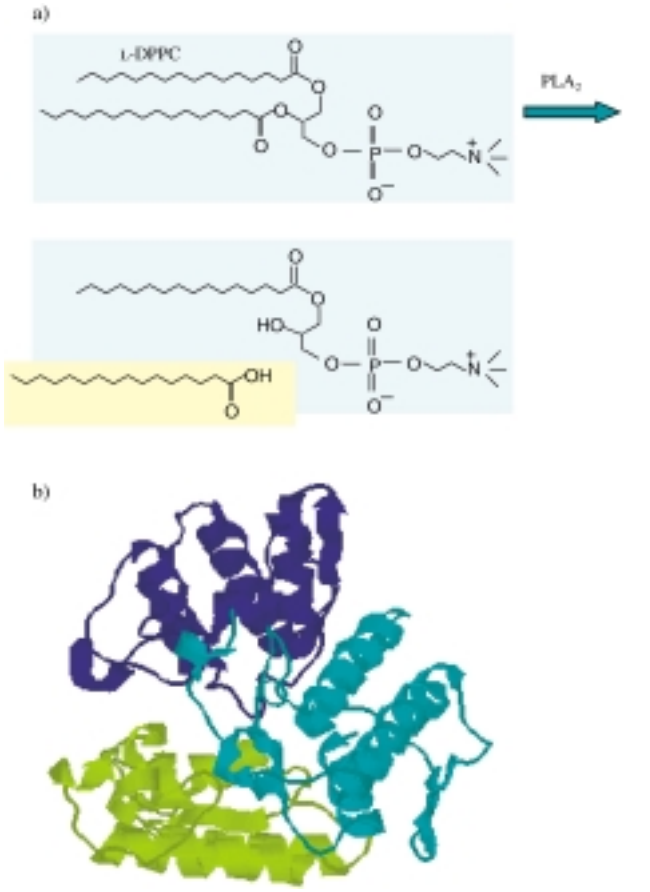

Figure 1. a) Schematic reoresentation of the hydrolysis of a L-DPPC monolayer by phospholipase A_2 (PLA_2). b) Representation of the PLA_2 structure.

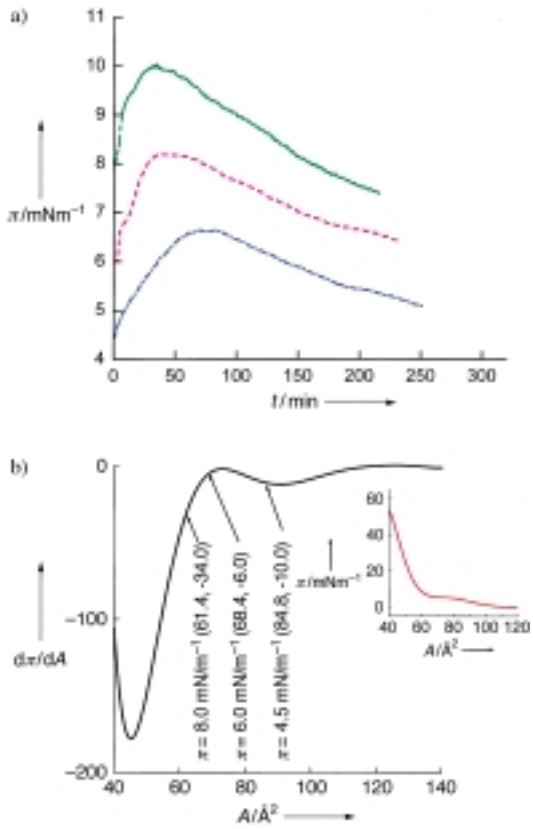

Figure 2. a) Variation of the surface pressure π as a function of time t. PLA_2 was injected into the subphase of the L-DPPC monolayer at different initial pressures: 4.5 (blue), 6.0 (red), 8.0 (green) mN m^{-1}. b) The slope dπ/dA of the DPPC isotherm (shown as an insert) as a function of molecular area A.

The BAM method opens a new possibility for the direct visualization of the texture of Langmuir monolayers.[3] It allows the long-range orientational order of a phospholipid in a monolayer to be characterized from the optical anisotropy induced by the tilted aliphatic chains.[4]

The results of the measurement of surface pressure as a function of time show that PLA_2 first causes an increase in surface pressure (Figure 2a). The pressure increase directly after the injection of the enzyme corresponds to the adsorption of PLA_2 onto the L-DPPC monolayer. The rate of the pressure increase (dπ/dt) can be related to the adsorption rate which is proportional to the area change dA/dt according to [Eq. 1].

$$\frac{d\pi}{dt} = \frac{d\pi}{dA} \times \frac{dA}{dt} \qquad (1)$$

An increase in the initial slope $(d\pi/dt)_0$ with pressure in the ratio 1.7:3.4:6.0 is observed whereas if dπ/dA is deduced from Figure 2b the ratio is 10:6:34. A comparison of the highest and lowest pressures at which the film is homogeneous shows that the pressure change is proportional to the change in dπ/dA and indicates a constant adsorption rate. For medium pressure, where the film is inhomogeneous, this proportionality does not hold because macroscopic and local compressibility deviate. In conclusion the adsorption rate is not determined by the free area of the surface of the film, but by transport in the bulk phase to the interface. The cleavage reaction that follows adsorption explains the low maximum surface pressure value at lower starting pressure and why the increase in the surface pressure is limited. On the other hand it was found that the surface pressure could reach the same maximum value, of about 10 mN m^{-1}, for a D-DPPC monolayer after PLA_2 adsorption but without enzymatic cleavage.[5] It was shown by FT-IR spectroscopy that of the reaction products the fatty acid remains at the interface and the lysophospholipid is partially dissolved into the solution.[6] Hence, the area demand of the lipid at around 50 Å^2 per molecule is reduced to that of 20 Å^2 for the fatty acid, and the surface pressure should decrease.

Polarization-modulated infrared reflection absorption spectroscopy[7] (PM-IRRAS) measurements of an L-DPPC monolayer revealed that PLA_2 has its maximum activity in the region where liquid-expended (LE) and liquid-condensed (LC) phases coexist region.[6] Micrographs of the morphology of L-DPPC monolayers attacked by PLA_2 in this phase-transition region show that the hydrolysis reaction starts at the interface between the liquid-expanded and condensed phases and progresses in the condensed phase.[8] Defects in the packing form the preferential reaction sites. Depending on the sample's history[9] the reaction starts either in the domain or at the edge. Domains damaged in the inner part, such as in the so called "pizza" type, were observed just 2 min after the

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injection of the PLA_2 ($C_{PLA2} = 0.975\ \mu g\,mL^{-1}$, $\pi_0 = 8.0\ mN\,m^{-1}$; Figure 3). The lysolipid is one of the reaction products and leaves the interface, this therefore increases the free space available. As a consequence, the fraction of the ordered region becomes smaller, which is reflected in the change of domains (Figure 3).

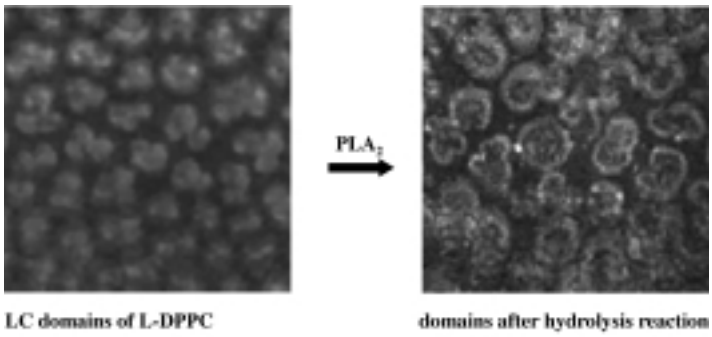

Figure 3. BAM images of a L-DPPC monolayer at the air/water interface before the injection of PLA_2 into the subphase and after the hydrolysis reaction.

The surface pressure decreases because of the loss of material at the interface, this again allows the penetration of the enzyme and a corresponding increase in pressure. The interplay of these factors results in the observed variation of surface pressure. It should be noted that in the absence of PLA_2 the size of the domains, not their number, changes with pressure. Enzyme addition in the absence of hydrolysis (D-enantiomer) does not affect the shape of the domain although the enzyme preferentially partitions at the domain boundaries.[5]

Figures 4a–4d show the change of the shape of L-DPPC domains after the injection of the enzyme at an initial pressure of $7.5\ mN\,m^{-1}$. The shape of the L-DPPC domains (dark area corresponds to condensed phase) starts to change 300 s after the injection of the PLA_2. The decrease in the area of the condensed-phase domains is attributed to the cleavage reaction. As shown in Figure 4b, there are two typical domain shapes: C- and O-form. The density of the C-form domains decreases drastically during the reaction, but their shape hardly changes. Therefore, we assume that the direction of hydrolysis in the C-form domains is along the curved domain boundaries. The long-range tilt orientation in the phospholipid monolayer has already been studied by BAM.[4] From the morphology of the pure L-DPPC monolayer it can be deduced that the orientation of the molecules in the elongated domains changes continuously along the boundaries and consequently follows the curvature of each segment.[10]

It can be deduced from the experiment, when the orientation of the molecules is considered, that the enzyme preferentially hydrolyzes the parts of the condensed domains with the same molecular chain orientation. If molecules with the same orientation (or reflectivity) are preferentially cleaved, C- or S-form domains are produced. The explanation is that the hydrolysis of molecules with the same orientation requires only a minimal change in the conformation of the enzyme during the cleavage. Figure 4b also displays O-shaped domains and indicates that the enzyme has hydrolyzed the central part of the domain, where, because of defects, the lipid molecules are less ordered. Thus, hollow O-form domains are produced. Finally, the domains can connect with each other to form a network.

A new kind of domain is observed about 80 min after the injection of the enzyme (Figure 4c). The shapes of these domains are very different from those of the DPPC monolayer and they have a relatively high reflectivity and are much brighter. The number of these domains increases with time and can be ascribed to the fact that the enzyme forms its own domains. This result is in good agreement with fluorescence microscopy studies on other proteins.[11] After 120 min, the mixed film is expanded and then recompressed. The domains now have a star shape (Figure 4d). To understand the transformation of the domains, we have to note that the presence of the enzyme causes a change of the line tension at the domain boundary[12] and that the monolayer is now a mixture of substrates and reaction products. One of these products remains at the interface as a fatty acid salt and probably forms phase-separated crystallites.

To show that the hydrolysis reaction first occurs in the condensed (LC) phase, the monolayer was compressed at a higher pressure. Figure 5 shows the BAM images taken during

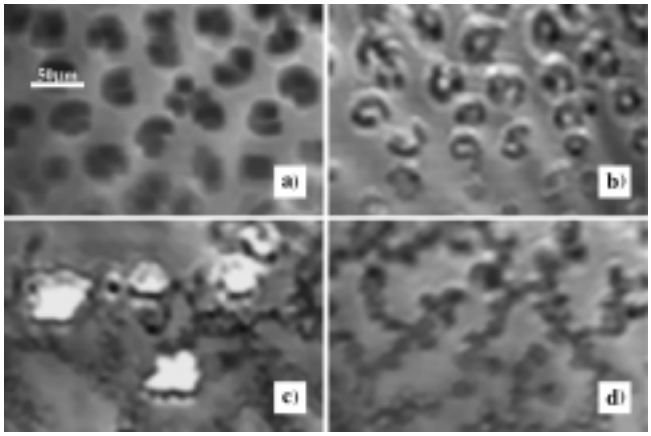

Figure 4. BAM images of an L-DPPC monolayer at the air/water interface during cleavage by PLA_2 with an initial surface pressure of $\pi_0 = 7.5\ mN\,m^{-1}$. For details see main text.

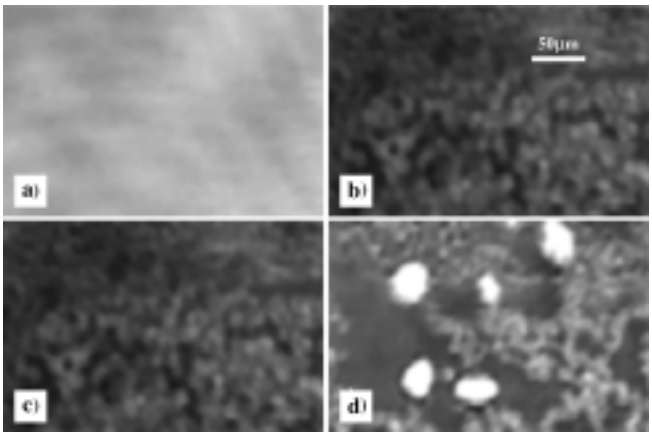

Figure 5. BAM images during the enzymatic reaction with an initial pressure of $15\ mN\,m^{-1}$. For details see main text.

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a reaction at an initial pressure of 15.0 mN m^{-1}. The monolayer is a homogeneous LC phase at this pressure (Figure 5a). After the injection of the enzyme, morphology changes caused by the hydrolysis reaction are observed (Figure 5b). Unlike the reaction in the biphase region, the cleavage reaction takes place randomly in the monolayer. A homogenous film is converted into a network, the holes in the monolayer gradually enlarge, and the shape of the edges becomes irregular (Figure 5c). At this higher pressure, the enzyme began to form its own domains only an hour after injection (Figure 5d).

The first step of the hydrolysis reaction is the surface recognition process and includes the adsorption of the enzyme onto the lipid surface. X-ray studies have shown that the tilt angle of the phospholipid molecules changes upon adsorption of specific enzymes.[5] This means that a change in the orientation of the lipid molecules is induced by the specific interaction between the lipid and the enzyme. As a result, the inner structure of the domains is different to that of the pure lipid. In addition to specific forces, long-range electrostatic forces can attract protein molecules to the domain boundary because the dipole moments normal to the surface of lipids and proteins are different.[11] This electrostatic force can also affect the orientation of the lipid molecules. In the interfacial recognition process at the start of the hydrolysis reaction both the enzyme as well as the phospholipid molecules could change their conformation.

In conclusion we have shown that enzyme penetration, the hydrolysis reaction, and the dissolution of the reaction products into the bulk phase determine the enzyme-catalyzed hydrolysis of phospholipids. These three processes can be separated by studying well-defined monolayers using BAM and film balance techniques. We anticipate that these results observed for the reaction of PLA_2 with L-DPPC can be generalized for a whole class of interfacial bioreactions. This is possible because the bilayer membrane is generally regarded to be a fluid phase. However, strong variations in the density and order of the membrane can lead locally to domain boundaries as discussed here. The amphiphile monolayers were used here as models to study stereospecific reactions in general, because they enable structural control and the microscopic follow up of reactions at interfaces. Although these monolayers are in an ordered state they allow access to the reactants through exchange with the adjacent bulk phase.

Experimental Section

A high-resolution Brewster angle microscope (Optrel, Germany) was mounted onto a computer-interfaced Langmuir trough (R&K, Germany) with a film balance. 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 afterwards through a frame grabber and processed by using special software to adjust the contrast and to correct the image distortion caused by observation at the Brewster angle. The surface tension was measured within 0.1 mN m^{-1} by the Wilhelmy method. The enzyme solution was injected into the subphase with a small syringe.

L-DPPC, D-DPPC, and PLA_2, from *Crotalus atrox Venom*, were purchased from Sigma and used without further purification. Chloroform (99%) was purchased from ACROS. In all experiments the subphase was an aqueous buffer solution at pH 8.9, which contained 15 mM NaCl, 5 mM $CaCl_2$, and 10 mM Tris. Experiments were performed at a temperature of $20.0 \pm 0.1\,^{\circ}C$. The concentration of the PLA_2 in the subphase was 0.714 mg mL^{-1}. All the water used in the experiments was purified by a Milli-Q system.

In the hydrolysis experiment, a 1 mM solution of L-DPPC in chloroform was spread on the surface of the buffer. The monolayer was compressed to the desired initial pressure and then the enzyme solution was injected into the subphase. The film pressure, and morphology of the formed domains were recorded simultaneously, the isotherm was recorded after 120 min.

In the experiment with D-DPPC monolayers, the monolayer was spread by the same method and slowly compressed to the coexistence region. Then the enzyme was injected into the subphase. After 60 minutes, the monolayer was expanded until the LC phase disappeared. The BAM images were recorded during the subsequent recompression. In all experiments the compression rate was 2.5×10^{-2} nm^2 per molecule per minute.

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