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Hydrolysis Reaction Analysis of L-α-Distearoylphosphatidylcholine Monolayer Catalyzed by Phospholipase A_2 with Polarization-Modulated Infrared Reflection Absorption Spectroscopy

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The hydrolysis reaction of L-α-distearoylphosphatidylcholine (DSPC) monolayers catalyzed by phospholipase A_2 (PLA_2) has been studied using polarization-modulated infrared reflection absorption spectroscopy (PM-IRRAS) with film balance measurements. The PM-IRRAS analysis provides quantitative information about the reaction efficiency at different surface pressures. It was found that the reaction efficiency of L-DSPC monolayer hydrolysis catalyzed by PLA_2 decreased with increasing surface pressure. At zero pressure (lift-off point), the hydrolysis reaction efficiency has the highest value of 45%. Increasing surface pressure leads to the decrease of the hydrolysis efficiency. Since the surface pressure is above 20 mN/m, the hydrolysis reaction nearly stopped. PM-IRRAS technique provides a powerful means to study the hydrolysis process catalyzed by phospholipase A_2 at the air/water interface.

Introduction

Phospholipase A_2 (PLA_2) is a calcium-dependent enzyme, which exists extensively in organisms. It can stereoselectively hydrolyze the sn-2 ester linkage of enantiomeric L-phospholipids to release fatty acids and lysophospholipids. Aggregated substrates, such as monolayers, lead to an activity 10 000-fold greater than that of monomers.[1−3] Correspondingly, two kinds of theories, the enzyme and the substrate theory, have been developed to explain the reaction mechanism.[4−7] A Langmuir monolayer is one of most suitable systems for investigating the cleavage process and the interfacial activity of phospholipase. Much work has been done to investigate the hydrolysis of L-α-dipalmitoylphosphatidylcholine (L-DPPC) monolayers catalyzed by phospholipase A_2 with various techniques.[8−10] It has been found that PLA_2 especially recognizes and preferentially hydrolyses condensed (LC) domains of L-DPPC monolayers.[8] The dependence of the reaction rate on surface pressure has been studied by means of polarization-modulated infrared reflection absorption spectroscopy (PM-IRRAS), which reveals that PLA_2 has its maximum activity in the LE/LC coexistence region of the L-DPPC monolayer.[9,10] Scanning force microscopy (SFM) and Brewster angle microscopy (BAM) experiments were also performed to investigate the morphology change due to hydrolysis by PLA_2. It demonstrates that the enzyme hydrolysis starts exclusively at membrane defects.[11−13] IRRAS has been regarded as a powerful method in "in-situ" investigation of enzyme interfacial reactions.[9,14−17] The reaction can be followed through the decrease of C=O band intensity, indicating the cleavage of the ester bond at the sn-2 position of the glycerol backbone of the lipids.[9,13,14,15,18]

In the present work, we investigated the hydrolysis process of an L-DSPC monolayer catalyzed by PLA_2 at different lateral pressures using PM-IRRAS.

Experimental Section

Chemicals. L-α-Distearoylphosphatidylcholine (DSPC, +99%), L-α-lysophosphatidylcholine, stearoyl (lysolecithin, stearoyl/LSPC, 99%), and stearic acid (octadecanoic acid, free acid/SA, 99%) were purchased from Sigma without further purification. Chloroform was obtained from ACROS (+99%). All phospholipids used were directly dissolved in chloroform to prepare 1 mM solutions for spreading phospholipid monolayers at the air/water interface. The fatty acid was dissolved in a mixed solvent of

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chloroform and methanol (volume ratio, 7:3). Methanol was HPLC grade with a purity of 99.9% from Promochem, Germany.

The phospholipase PLA_2 (Crotalus Atrox Venom) was obtained from Sigma. To keep the enzyme at its highest activity, the pH value of an aqueous buffer solution, which contains 150 mM NaCl, 5 mM $CaCl_2$, and 10 mM Tris was adjusted exactly to 8.9 by using 1 M HCl solution. The water used was purified by a Milli-Q system, and the resistance of the Millipore water is 18.2 MΩ cm. All the experiments were performed at 25 ± 0.1 °C.

Monolayer Preparation for Hydrolysis Experiments. A 1 mM solution of phospholipid/chloroform was spread on the subphase of pH 8.9 buffer solution. After solvent evaporation, the monolayer was compressed at a rate of around 0.05 $Å^2$/(molecule·s). As the surface pressure reached the required value, the compression was stopped. Then, from both sides of the trough barriers, the enzyme solution was injected into the subphase underneath the monolayer by a microsyringe. Changes of surface pressure and morphology were recorded simultaneously. The surface pressure was recorded by the Wilhelmy method with an error of ±0.1 mN/m.

Polarization-Modulated Infrared Reflection Absorption Spectroscopy. To determine the composition of the monolayer quantitatively, all monolayers for PM-IRRAS experiments were spread in a special Langmuir trough, which provides a nonmembrane region on one side of the barrier for the IR-spectrum measurement of the pure subphase. The optical setup consists of an IR source, a Michelson interferometer from a Bruker IFS66 (Bruker, Karlsruhe, Germany) spectrometer, and an external reflection unit. At the exit of the interferometer, a mirror directs the IR beam into the adjustable arm of the reflection unit. It passes both a polarizer (KRS5, Specac, Orpington, Great Britain) and a photoelastic modulator (ZnSe, Type II, Hinds) and is then focused by a paraboloidal mirror onto the water surface. The trough is placed in a sealed box fitted with BaF_2 windows. The reflected intensity is focused by a ZnSe lens onto a liquid nitrogen cooled MCT detector. The detected intensity is double modulated, first by the interferometer (300−1200 Hz) and then by a photoelastic modulator (73.9 kHz). The electronic setup used is designed to obtain two signals ($R_p - R_s$) and ($R_p + R_s$), where R_p and R_s represent the reflectivities for p- and s-polarization, respectively. Whereas one part ($R_p + R_s$) is treated only by band-pass filtering (300−1200 Hz, Stanford research system, Model SR 650), the other part ($R_p - R_s$) is first band-pass filtered around 73.9 kHz, then demodulated (EG&G Lock-In amplifier, Model SR 650), and band-pass-filtered (300−1200 Hz) again. The signals are multiplexed and connected with the standard electronics of the spectrometer. The resulting interferogram is amplified, digitized (16 bit AD-board), and Fourier transformed with level 2 of zero filling and a triangle apodization function (OPUS software). We chose the same base line and the same integration model to integrate the area of the bands with the provided software of the PM-IRRAS instruments.

The IR spectra were collected using 200 scans at 4 cm^{-1} resolution with a total scanning time of about 5 min. Only anisotropic absorptions contribute to the PM-IRRAS signal S[19]

$$S = C\frac{R_p - R_s}{R_p + R_s}J_2(\phi) \quad (1)$$

where C is a constant of the electronical setup and $J_2(\phi)$ the second-order Bessel function describing the wavenumber dependence of dephasing.

Isotropic absorption by the water vapor or the subphase is not detected. Normalized difference spectra

$$\Delta S = \frac{S_d - S_0}{S_0} \quad (2)$$

are given, with S_0 the signal of the bare water surface and S_d the signal of the film-covered surface. The angle of incidence was set to 74°. The spectra shown are raw spectra with no base-line correction or smoothing required. In all spectra, a broad negative band around 1650 cm^{-1} appears. This band arises from the different reflectivities of covered and bare water surfaces and is due to the wavelength dependence of the complex refractive index of water.[20] A restructuring of water molecules at the interface may also explain the very strong intensity of this band.[19,20]

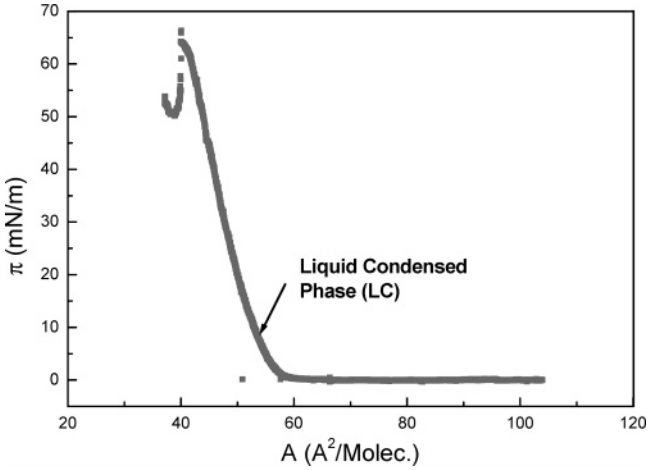

Figure 1. π−A isotherm of pure L-DSPC monolayer on the buffer solution at pH 8.9.

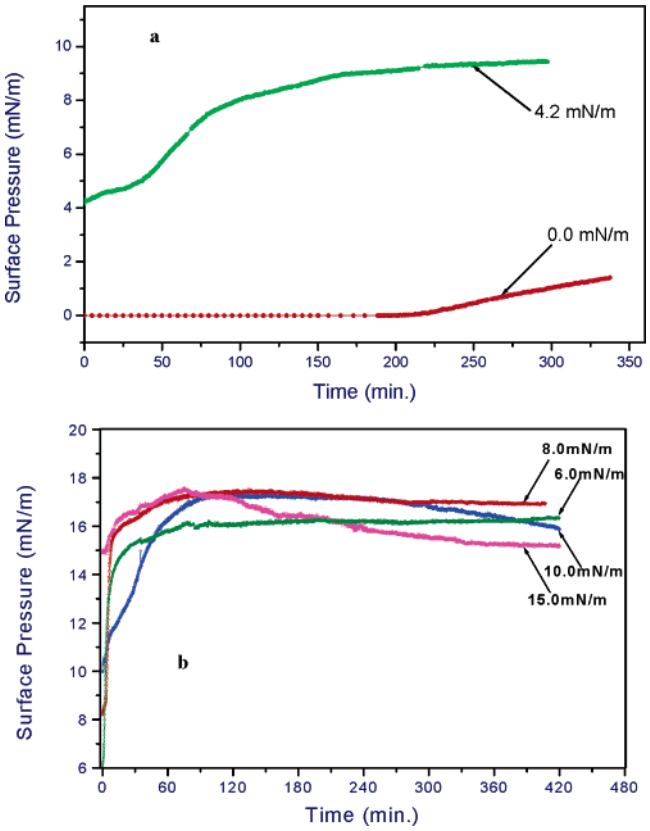

Figure 2. Curves of surface pressure π with time t for L-DSPC monolayer hydrolysis by PLA_2 at different initial surface pressures, 0, 4, 6, 8, 10, and 15 mN/m, respectively.

Results and Discussion

Figure 1 shows the isotherm of an L-DSPC monolayer on buffer at 25 °C. At zero pressure and molecular areas above 58 $Å^2$, the monolayer is in a two-phase coexistence region between a gas-analogous and a condensed state. Below molecular areas of 58 $Å^2$, the L-DSPC monolayer

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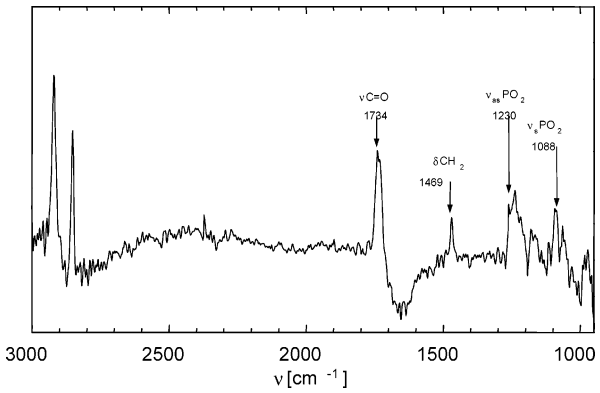

Figure 3. PM-IRRAS spectrum of a pure L-DSPC monolayer at $\pi = 40$ mN/m.

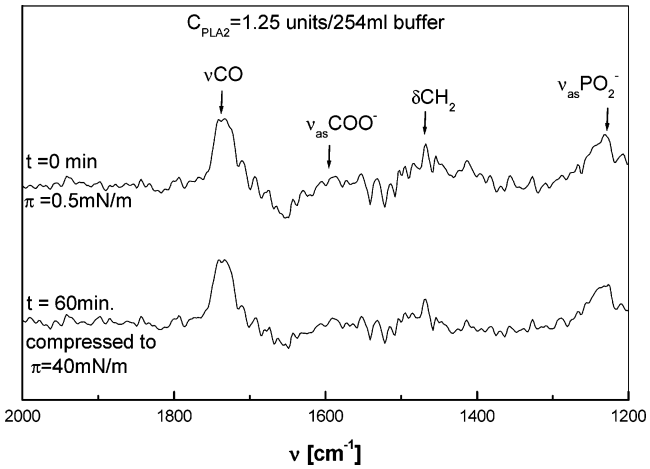

Figure 4. PM-IRRAS spectra of L-DSPC monolayer before and after injection of PLA_2 with the enzyme concentration in the subphase of 21.6 ng/mL at the surface pressure of 0.5 mN/m. After 60 min, the monolayer was compressed to $\pi = 40$ mN/m for the PM-IRRAS measurements.

is fully condensed (LC).[21] The $\pi-t$ curves in Figure 2a,b present the surface pressure changes of L-DSPC monolayers with time during hydrolysis at different initial surface pressure π_0 = 0, 4, 6, 8, 10, and 15 mN/m, respectively. It takes a certain time for the surface pressure to reach the maximum. Especially when the reaction starts at low surface pressures, e.g., 0 and 4 mN/m, the surface pressure keeps on increasing even after 5−6 h and does not reach its maximum yet (Figure 2a). At higher surface pressure it takes less time to reach the maximum value (Figure 2b). At $\pi_0 = 6$ mN/m, the surface pressure can reach 16 mN/m, while, starting at 8 mN/m, the surface pressure has a maximum value of 17 mN/m. This may be ascribed to the fact that PLA_2 partly penetrates into the monolayer and that Lyso-SPC prefers to stay in the interface due to the longer chain and therefore smaller solubility in water.

To evaluate the pressure dependence of the hydrolysis reaction in L-DSPC monolayers quantitatively, PM-IRRAS was utilized to analyze the reactants and products at the interface. Figure 3 shows a PM-IRRAS spectrum of a pure L-DSPC monolayer at 40 mN/m. The water vapor bands are completely compensated. It should be noted that the interfacial carbonyl ester C=O stretching vibration of L-DSPC molecules appears at 1734 cm^{-1} and a CH_2-scissoring mode is found at 1469 cm^{-1}. Bands at 1230 and 1088 cm^{-1} are due to a heterogeneous population of

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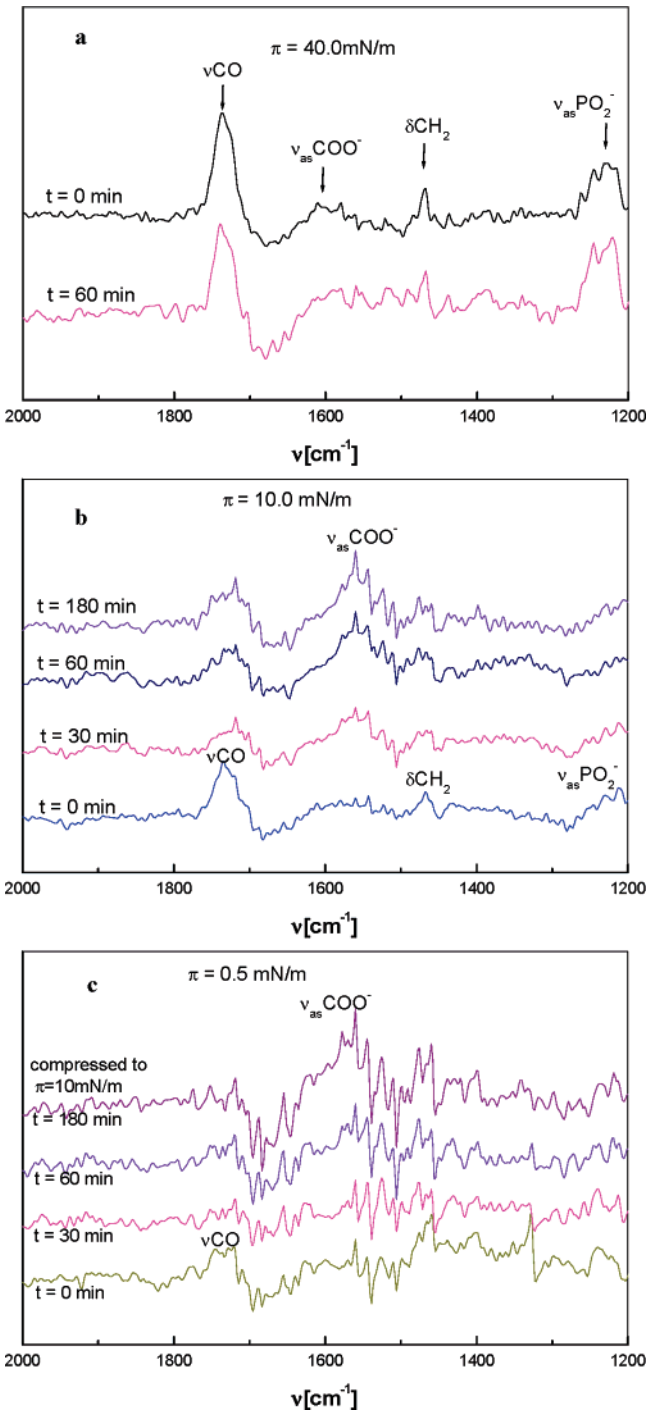

Figure 5. PM-IRRAS spectra of L-DSPC monolayer hydrolysis by PLA_2 with the enzyme concentration in the subphase of 173 ng/mL. The surface pressures were kept at different initial surface pressure for the hydrolysis reaction. (a) $\pi = 40$ mN/m and (b) $\pi = 10$ mN/m for 180 min, respectively, and then the monolayer was compressed to $\pi = 40$ mN/m for the PM-IRRAS measurements. (c) $\pi = 0.5$ mN/m for the hydrolysis reaction of 180 min, and then the monolayer was compressed to $\pi = 10$ mN/ m for the PM-IRRAS measurements.

hydrated and nonhydrated phosphate groups. The intensities of the bands depend on the lateral density of the molecules and on the orientation of the transition moments in the plane of incidence. The ester band at 1734 cm^{-1} exhibits an almost linear relationship between the integrated band intensity and the molecular area.[9]

Since PLA_2 stereoselectively hydrolyzes the sn-2 ester linkage of L- phospholipids to produce fatty acid and

lysolipid, the hydrolysis can be quantified by measuring the intensity of the ester band at 1734 cm^{-1} by PM-IRRAS. At the same time, the carboxylate bands should increase with time. The band at 1540 cm^{-1} represents an unassociated carboxy group and the band at 1578 cm^{-1} a Ca^{2+} associated carboxy group in highly condensed monolayer state. Here in highly condensed monolayer state the band at 1562 m^{-1} is shifted to 1578 m^{-1} and must be caused by an interaction between protein PLA_2 and the carboxylate group of produced fatty acid.[9,14]

Figure 4 shows the PM-IRRAS spectra of L-DSPC monolayers before and after injection of PLA_2 with an enzyme concentration in the subphase of 1.25 units/(254 mL) (C_{PLA_2} = 21.6 ng/mL). The lateral pressure was set to 0.5 mN/m. As one can see, there are no changes of the spectra after 60 min. Correspondingly no hydrolysis reaction occurred. This may be ascribed to the low enzyme concentration. The $\pi\angle t$ curves in Figure 2 show similar results. Spectra changes of L-DSPC monolayers with reaction time were recorded at different initial surface pressures of 40, 10, and 0.5 mN/m for enzyme concentrations increased to 10 units/(254 mL) (C_{PLA_2} = 173 ng/mL). However, at 40 mN/m we did not observe any change, as presented in Figure 5a. This indicates that at such a high lateral surface pressure the hydrolysis reaction cannot occur. At 10 mN/m, the ester band intensity at 1734 cm^{-1} decreases quickly within the first 30 min and the fatty acid bands increase correspondingly (Figure 5b). It directly reflects the formation of hydrolysis products. Figure 5c shows the obvious change of the spectra after the injection of enzyme into the subphase at 0.5 mN/m. Immediately after enzyme injection a pronounced decrease of the ester band can be seen. It is noted that due to the faster hydrolysis rate and the space restriction of the instruments we are unable to compress the monolayer to 40 mN/m after 180 min of hydrolysis at 0.5 mN/m. Thus the monolayer can only be compressed to 10mN/m for performing the PM-IRRAS measurements.

Figure 6 shows the amount of hydrolyzed monolayer substrate. This amount decreases with increasing surface pressure. At almost zero pressure, where the packing density in the condensed phase is the lowest, the hydrolysis yield has its maximum value of 45%. When the surface pressure increased to around 20 mN/m, the hydrolysis reaction efficiency was reduced to nearly zero. It means that at high packing densities the hydrolysis reaction is almost inhibited.

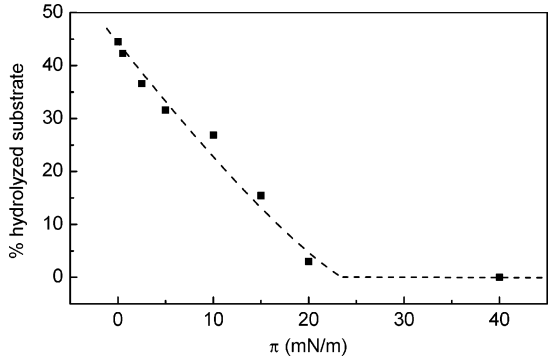

Figure 6. Percentage of substrate hydrolyzed within 60 min. The percentage was evaluated by using the integrated intensity of the ester band at 1734 cm^{-1} measured at 40 mN/m and compared with calibration curves obtained for defined ternary mixed monolayers.

Conclusions

The hydrolysis reaction of L-phospholipid monolayers at the air/water interface catalyzed by PLA_2 was studied by using polarization-modulated infrared reflection absorption spectroscopy to quantitatively determine the hydrolysis yield in dependence on the surface pressure. It was found that the reaction efficiency of the L-DSPC monolayer hydrolysis catalyzed by PLA_2 decreases monotonically with increasing surface pressure. The lysolecithin as one of the hydrolysis products remains obviously in the monolayer even after long reaction times, indicating that it has a high surface activity. At the lowest packing density of the monolayer, i.e., at zero pressure, it is found that the hydrolysis reaction efficiency is the highest. At surface pressures above 20 mN/m, no hydrolysis reaction was observed.

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