Dipalmitoyl-Phosphatidylcholine/Phospholipase D Interactions Investigated with Polarization-Modulated Infrared Reflection Absorption Spectroscopy

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ABSTRACT The hydrolysis of 1,2-dipalmitoylphosphatidylcholine (DPPC) catalyzed by *Streptomyces chromofuscus* phospholipase D (PLD) has been investigated using monolayer techniques and polarization-modulated infrared absorption reflection spectroscopy. The spectroscopic analysis of the phosphate groups provides a quantitative estimation of the hydrolysis yield. The hydrolysis kinetics was investigated in dependence on the phase state of the lipid monolayer. It was found that PLD exhibits maximum activity in the liquid-expanded phase, whereas PLA_2 has its activity maximum in the two-phase region. A lag phase was observed in all experiments indicating that small amounts of the hydrolysis product 1,2-dipalmitoylphosphatidic acid (DPPA) are needed for initiating the fast hydrolysis reaction. Higher concentrations of DPPA inhibit the hydrolysis. The critical inhibition concentration of DPPA is a function of the monolayer pressure.

INTRODUCTION

Phosphatidylcholine phosphohydrolase (PLD) belongs to a lipolytic enzyme subclass that catalyzes the hydrolysis of phospholipids (e.g., 1,2-dipalmitoylphosphatidylcholine (DPPC)) to phosphatidic acids (e.g., 1,2-dipalmitoylphosphatidic acid (DPPA)) and produces a free polar group (e.g., choline).

PLD activity is present in a wide variety of cell types including blood platelets, fibroblasts, muscle cells, and others. Studies of PLD activity in intact cells and tissue have clearly established a role of the enzyme in cellular signal transduction across membranes (Singler et al., 1997). PLD activation has been correlated with a number of cellular events. Moreover, the hydrolysis product DPPA, a second messenger molecule, plays an important role in mitogenic signaling pathways (Salmon and Honeyman, 1980).

Many different methods have been used to determine PLD enzymatic activity (Eldar et al., 1993; Horwitz and Davies, 1993; Imamura and Horiuti, 1978; Liscovitch et al., 1993). The monomolecular film technique opens new possibilities for studying the hydrolysis process (Abousalham et al., 1996; Dahmen-Levison et al., 1998a; Kondo et al., 1994; Pieroni et al., 1990; Quarles and Dawson, 1969; Ransac et al., 1991; Slotboom et al., 1976; Verger et al., 1976). This technique allows varying such physical-chemical parameters of the interface as molecular density, lateral pressure, surface potential, or ionic conditions. Therefore, it provides the possibility to study the influence of the chemical structure of the substrate as well as of its phase state on yield, velocity, and kinetics of the hydrolysis process.

A potentially suitable method for obtaining information about enzymatic processes on a molecular scale is infrared spectroscopy (Dicko et al., 1998; Gericke and Hühnerfuss, 1994; Dahmen-Levison et al., 1998b; Mantsch and McElhaney, 1991), which provides insight into the conformation and orientation changes of both acyl chains and the polar headgroup.

In the present study we have investigated the PLD-catalyzed hydrolysis of DPPC using the Langmuir technique and polarization-modulated infrared reflection absorption spectroscopy (PM-IRRAS). The kinetics of the hydrolysis process has been studied by the analysis of the PO_2^- stretching vibration bands. A quantitative estimation of PLD hydrolysis yield in dependence on the substrate structure was obtained.

MATERIALS AND METHODS

DPPC, DPPA, and PLDs (from *Streptomyces chromofuscus* and cabbage) were purchased from Sigma Chemical Co. (St. Louis, MO) and used without further purification. The phospholipids were dissolved in chloroform to prepare 1 mM solutions. The subphases were aqueous buffer solutions with 50 mM $CaCl_2$, 150 mM NaCl, and 10 mM Tris at pH 8 (for PLD from *S. chromofuscus*) and 50 mM $CaCl_2$, 100 mM $NaCH_3COO$ at pH 5.6 (for the cabbage PLD). The calcium concentration needed for the stimulation of the hydrolysis reaction depends on the type of PLD used. In all cases, saturation was reached above a concentration of 20 mM (Quarles and Dawson, 1969; Imamura and Horiuti, 1978; Kondo et al., 1994). To be well above this limiting concentration and to exclude any concentration influence, a high $CaCl_2$ concentration has been used in our experiments.

Water was purified with a Millipore desktop system, leading to a specific resistance of 18.2 MΩcm. PLD was dissolved in the same buffer solution as used as a subphase.

The film balance was built by R&K (Wiesbaden, Germany) and equipped with a Wilhelmy-type pressure measuring system. The phospholipid solution was spread at the air-water interface. After 10 min the monolayer was compressed with a velocity of 15 $Å^2$/molecule min to a lateral pressure of 40 mN/m and a first control spectrum was measured. At this pressure, the enzyme solution was injected and carefully stirred un-

Received for publication 7 January 2000 and in final form 23 October 2000.

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0006-3495/01/02/749/06 $2.00

derneath the monolayer. This surface pressure was used because our PM-IRRAS experiments have shown that no hydrolysis occurs during 24 h. Then the film was expanded to a chosen pressure at which the kinetics of the hydrolysis process was investigated by PM-IRRAS. The pressure was kept constant during the reaction by an automatically moving barrier. After finishing the hydrolysis process the monolayer was again compressed to 40 mN/m and a final control spectrum was measured. Then the film was expanded and the resulting isotherm of the mixture of hydrolysis product (DPPA) and substrate (DPPC) was obtained.

PM-IRRAS spectra were recorded using a Bruker IFS66 spectrometer (Bruker, Karlsruhe, Germany) equipped with an MCT detector. A detailed description of the PM-IRRAS setup and the experimental procedure has already been given elsewhere (Buffeteau et al., 1991). The setup consists of an IR source, a Michelson interferometer and an external reflection unit. The infrared radiation intensity was modulated by the interferometer and polarized with a ZnSe polarizer. The beam was then passed through a ZnSe photoelastic modulator, which modulated it between polarization in the plane of incidence (p) and polarization perpendicular to this plane (s) with a fixed frequency of 73 kHz. From the detected intensity (using electronic filtering and demodulation) the two signals, $(R_p - R_s)$ and $(R_p + R_s)$, were obtained. Only anisotropic absorptions contribute to the PM-IRRAS signal $S \approx (R_p - R_s)/(R_p + R_s)$. Normalized signals were obtained using the following expression: $\Delta S = (S_d - S_o)/S_o$, where S_d and S_o are the signals in the presence and absence of a monolayer, respectively. The angle of incidence of the infrared beam with respect to the surface normal was 74°. Spectra were recorded with a spectral resolution of 4 cm^{-1} and collected using 200–400 scans during 5–10 min.

RESULTS AND DISCUSSION

Spectroscopic estimation of fraction of substrate hydrolyzed

An IR spectrum of a DPPC monolayer recorded at 20°C and at a pressure of 40 mN/m is shown in Fig. 1. The spectral regions between 900 and 1800 cm^{-1} and 2800 and 3000 cm^{-1} are very important for obtaining information on the orientation and conformational order of hydrocarbon chains (νCH_2), on the subcell structure (δCH_2), on the hydration, H-bonding, and ion binding (e.g., νCO, νPO_2^-), as well as on the conformation of the headgroup (νCN^+C, νPO_2^-) (Hunt et al., 1989; Mendelsohn et al., 1995).

The most convenient band to follow the hydrolysis reaction would be the vibration of νCN^+C because it is absent in the spectrum of the reaction product DPPA. However, this band has not been used because the response of the detector drastically decreases at wave number smaller than 970 cm^{-1}. The signal/noise ratio decreases especially in this region due to evaporation of water during the reaction time. Additionally the intensity of this band is weak and decreases during the hydrolysis. Hence other characteristic vibrational bands have to be identified. Therefore, the spectra of pure DPPC as the substrate and of pure DPPA as the hydrolysis product have been recorded on different subphases (Fig. 2). Two different buffer solutions, which provide optimal conditions for different PLDs, have been used in the experiments. Fig. 2 shows that DPPC and DPPA can be distinguished by the spectroscopic parameters of carbonyl and phosphate vibrational bands: νCO, $\nu_s PO_2^-$, and $\nu_{as} PO_2^-$. The νCO band has a pronounced asymmetry on the low-frequency side. It consists of two overlapping components, one at 1740 cm^{-1} and another at ~1726 cm^{-1}. The high-frequency component of this band is assigned to the non-hydrogen-bonded (free) carbonyl group and the lower-frequency component to the hydrogen-bonded carbonyl group. An increase of the intensity of the free carbonyl band can be seen for DPPA compared with DPPC. Therefore, a high-frequency shift of the overall CO band (Fig. 2) was observed. This can be explained with a tighter packing of DPPA at 40 mN/m and hence with a reduction of the hydration of the carbonyl group.

An even more pronounced difference between the spectra of DPPA and DPPC is observed for the integral intensities of bands in the region of the phosphate stretching vibrations (Fig. 2). Also high-frequency-shifted antisymmetric stretching phosphate vibrations were found for DPPA. This can be explained by a partial dehydration of these groups due to the interaction with Ca^{2+} ions.

As one can see from Fig. 2, the integral intensities of the symmetric and the antisymmetric PO_2^- vibrations for DPPA strongly depend on the ionic environment and the pH of the buffer. The conformation of the negatively charged headgroup of DPPA and therefore the angle between the orientation of the dipole moment of the PO_2^- group and the monolayer plane is obviously different for various pH and ionic compositions. This leads to an intensity increase of the symmetric and a decrease of the antisymmetric phosphate vibrations. Contrary to DPPA, the parameters of the vibrational bands of DPPC do not change as a function of pH and buffer composition.

The antisymmetric PO_3^{2-} vibration, observed in organophosphorus compounds (Thomas and Chittenden, 1970) and dihexadecylphophatidic acid (Laroche et al., 1991), has a frequency of 1084 cm^{-1}. Therefore, it can give a contribution to the integral intensity of the symmetric PO_2^- vibration band centered at 1090 cm^{-1}. Actually the phosphate headgroup bears a single negative charge (Cevc, 1987; Garidel and Blume, 2000) and has a C_{2v} symmetry group at pH 8. The presence of Ca^{2+} can give rise to the removal of a proton and the delocalization of two negative charges in the headgroup as suggested in Garidel and Blume, 2000, and Laroche et al., 1991. In this case a C_{3v} group is present and the PO_3^{2-} vibrations can be observed. However, the replacement of the proton of the P-OH group is only partially because the antisymmetric PO_2^- vibration can be seen on all buffers used.

The phosphate vibrations were chosen for the quantitative estimation of the amount of hydrolysis product. The vibrational spectra (Fig. 3 *a*) were measured for monolayers of different DPPA/DPPC mixtures on the buffer with pH 8 chosen for the *Streptomyces* PLD. As one can see in Fig. 3 *a*, the integral intensity of the $\nu_s PO_2^-$ and $\nu CO(P)$ decreases and that of the

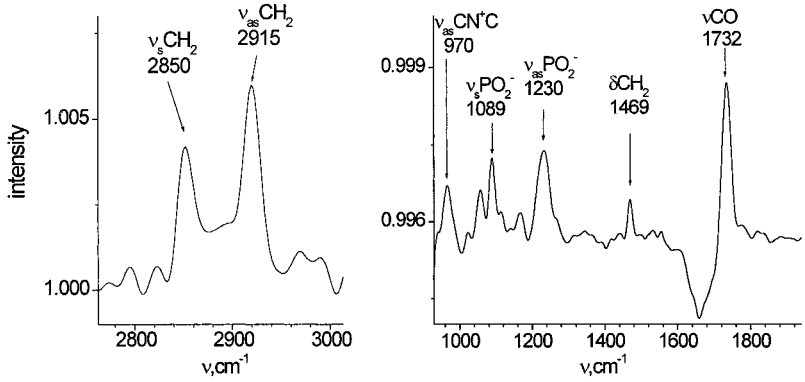

FIGURE 1 PM-IRRAS spectra of a DPPC monolayer at a surface pressure of 40 mN/m.

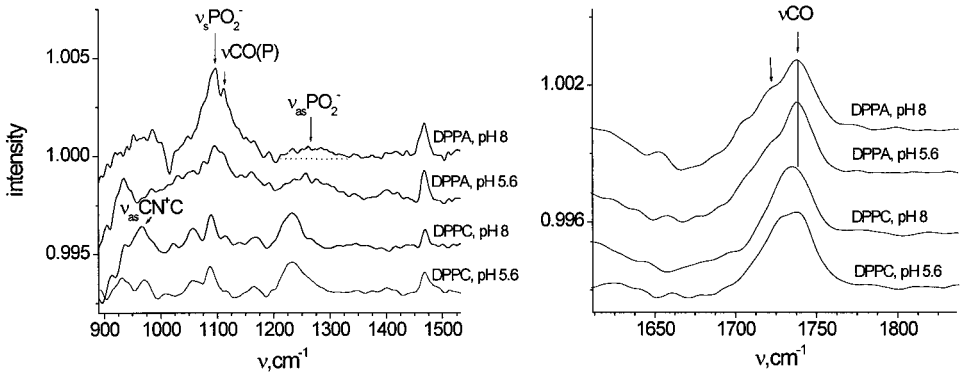

FIGURE 2 PM-IRRAS spectra of DPPC and DPPA monolayers on different subphases (aqueous buffer solutions with 50 mM $CaCl_2$, 150 mM NaCl, 10 mM Tris at pH 8 or with 50 mM $CaCl_2$, 100 mM $NaCH_3COO$ at pH 5.6). The phosphate and carbonyl stretching regions are shown.

$\nu_{as}PO_2^-$ vibrations increases continuously with increasing percentage of DPPC. Additionally, the carbonyl vibrations shift to higher frequencies. From Fig. 3 *b* follows that the integral intensities of the ($\nu_sPO_2^-$ + νCO(P)) and $\nu_{as}PO_2^-$ vibrations linearly depend on the mole fraction of DPPC. Therefore, it was concluded that the headgroup conformation of DPPA remains the same in all mixtures and the intensity of these vibrations depends only on the quantity of DPPA. Such linearity is expected for ideal mixtures or phase-separated systems. In many cases phase separation was observed in phosphatidylcholine/phosphatidic acid dispersions (Kouaouci et al., 1985; Garidel et al., 1997; Garidel and Blume, 2000). However, there are also observations that mixtures of zwitterionic and ionic lipids retain their miscibility in the presence of Ca^{2+} in monolayers (Flach et al., 1993). This graph allows us to estimate the mole fraction of DPPA in mixed monolayers. It can also be cross-checked by using the CO bands as a standard because their intensity does not noticeably change for different mixtures.

Kinetics of DPPC monolayer hydrolysis

The kinetics and the yield of the enzymatic hydrolysis of DPPC were investigated as a function of the type of enzyme, of its quantity, and of the substrate structure using PM-IRRAS and the film balance technique. It was found that cabbage PLD only partially hydrolyzed the DPPC molecules in the monolayer even at optimal conditions (Kondo et al., 1994), whereas PLD from *Streptomyces* almost completely hydrolyzed DPPC. For this reason, *Streptomyces* PLD was chosen for subsequent kinetic investigations.

The fraction of substrate hydrolyzed during 100 min using 25 and 255 units of PLD was studied at different surface pressures: 1) below the plateau region in the liquid-expanded phase LE, 2) in the coexistence region of LE and a liquid-condensed (LC) phase, 3) in the LC phase. Fig. 4 shows isotherms of DPPC and their changes during the hydrolysis catalyzed by PLD (25 units) at different pressures (1.5 mN/m and 20 mN/m). The enzyme was injected at 40 mN/m (A in Fig. 4), a control spectrum was taken and the monolayer was expanded to the reaction pressure (B in Fig. 4). The area per molecule is decreasing during the hydrolysis (B → C in Fig. 4). PM-IRRAS spectra were continuously taken during the reaction. An increase of the area, probably caused by penetration of the phospholipase into the monolayer, was only occasionally detected in some experiments, presumably because the catalytic amount of the enzyme is so small that any increase in area would be masked by the area decrease due to the reaction. The area decrease (from B to C) can be explained by the smaller molecular area of the hydrolysis product DPPA compared with DPPC at the same surface pressure. The velocity of the area decrease depends on the amount of PLD used and the structure of the monolayer. After 100 min of hydrolysis catalyzed by 25 units of PLD or after 30 min using 255 units, the monolayer was compressed to 40 mN/m (D in Fig. 4) to compare the monolayer spectra at well defined identical conditions. In the last step, the expansion curve was recorded (D → E → F). The differences in the isotherms already indicate that a different amount of DPPC is hydrolyzed during the reaction. DPPA exhibits a fully condensed isotherm, whereas DPPC undergoes a transition from a liquid-expanded to a condensed state characterized by a plateau region. Therefore, the remaining plateau in the isotherm after the reaction at 20 mN/m indicates that there is still a considerable concentration of DPPC in the mixed monolayer. The final spectra at 40 mN/m show different intensity distributions of the symmetric and antisymmetric phosphate vibrations, which are used for the quantitative evaluation of the DPPA content after the hydrolysis. The yield (fraction of hydrolyzed substrate) as a function of pressure is presented in Fig. 5. One realizes a monotonic decay but with an extended plateau region between 4 and 10 mN/m. Fig. 6 shows the kinetics of the hydrolysis at various selected lateral pressures. One observes a linear increase followed by a plateau. As one can see from Figs. 5 and 6 the PLD exhibits maximum activity at the lowest pressure (1.5 mN/m) investigated. At this pressure, the monolayer is in a liquid-expanded state. The amount of PLD used has

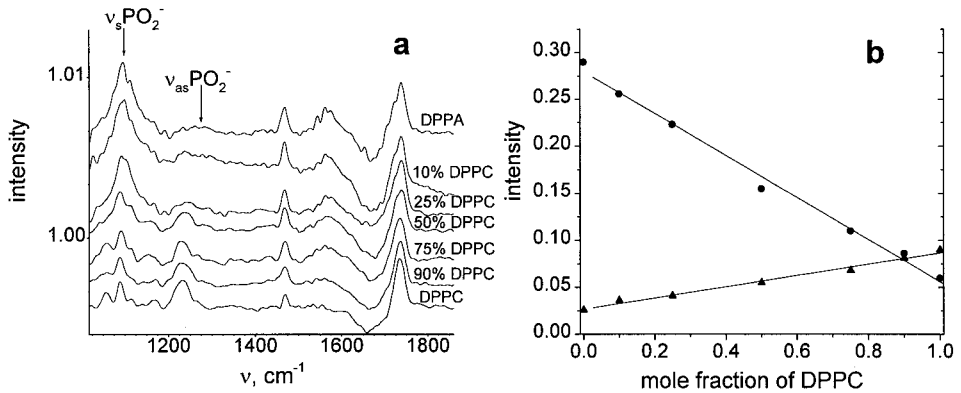

FIGURE 3 (*a*) PM-IRRAS spectra of different DPPA/DPPC mixtures at a surface pressure of 40 mN/m. The amount (mol %) of DPPC in the mixtures is indicated. The subphase was an aqueous buffer solution with 50 mM $CaCl_2$, 150 mM NaCl, 10 mM Tris at pH 8. (*b*) Integral intensity of the phosphate stretching vibrations as a function of the DPPC mole fraction in mixtures with DPPA. ●, sum of symmetric PO_2^- and CO(P) vibration bands; ▲, antisymmetric PO_2^- vibration band.

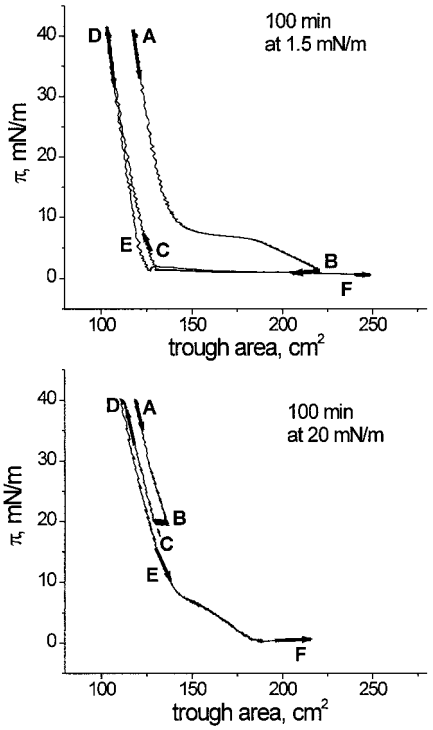

FIGURE 4 Pressure-area isotherms of DPPC monolayer on the buffer with pH 8 during and after the hydrolysis reaction catalyzed by 25 units of PLD (*S. chromofuscus*). The PLD was injected underneath the compressed (40 mN/m) monolayer, which was afterwards expanded to the reaction pressure (1.5 mN/m or 20 mN/m). After 100 min the monolayer was first compressed to 40 mN/m and then fully decompressed.

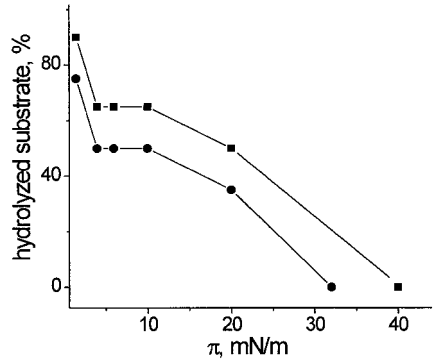

FIGURE 5 Hydrolysis yield (percentage of hydrolyzed DPPC) catalyzed by 25 (●) and 255 (■) units of PLD (*S. chromofuscus*) after finishing the hydrolysis process (after 100 min) versus reaction surface pressure. The subphase was an aqueous buffer solution with 50 mM $CaCl_2$, 150 mM NaCl, 10 mM Tris at pH 8.

only a small influence on the final result (Fig. 5). This indicates that 25 units are close to and 255 units are well above the saturation concentration (Quarles and Dawson, 1969).

The value of the optimal hydrolysis pressure seems to be a characteristic parameter of both the enzyme and the lipid (Slotboom et al., 1976; Verger et al., 1976; Pieroni et al., 1990; Ransac et al., 1991; Dahmen-Levison et al., 1998b). Comparing different phospholipases, interacting at different positions in the hydrophilic and hydrophobic regions, allows us to get a more detailed insight into the mechanisms of hydrolysis. For example, phospholipase A_2 (PLA_2), which hydrolyzes the *sn*-2 ester linkage of DPPC, has maximum activity in the two-phase coexistence region between the liquid-expanded and condensed phases for monolayers (Grainger et al., 1989; Dahmen-Levison et al., 1998b). PLA_2 adsorption has a strong influence on the monolayer structure (Dahmen-Levison et al., 1998a). These findings indicate the importance of a pre-orientation of DPPC molecules for the PLA_2-catalyzed hydrolysis. In contrast to PLA_2, PLD acts in the hydrophilic region of the phospholipid. The observation that the maximum activity of PLD is found in a more disordered phase indicates that fluidity and defects in the monolayer structure are more important than an adsorption-induced pre-orientation of the substrate.

Yield and velocity of the DPPC hydrolysis are remarkable at low and intermediate pressures and decrease drastically at higher lateral pressures (Fig. 5). This effect can be explained as follows. At high lateral pressures, the phospholipid molecules are tightly packed and the penetration of the active center of the enzyme is not possible, whereas at lower pressures the enzyme penetrates more easily into the monolayer. The constant hydrolysis yield and velocity occurring between 4 and 10 mN/m may be the result of two parallel influences: 1) the increase of surface concentration of the substrate favors an increase of the catalysis velocity, and 2) on other hand, the increase of the surface pressure decreases gradually the penetration ability of the enzyme. Indeed, it is widely assumed that phospholipases cannot hydrolyze the substrate unless at least partial penetration of the enzyme into the lipid layer occurs. If, for example, the monolayer pressure is too large the phospholipases may not be able to penetrate, because the free energy penalty associated with the area increase would be too large. Such threshold pressure was observed for different phospholipases (Quarles and Dawson, 1969; Demel et al., 1975; Hirasawa et al., 1981). The threshold pressure depends on the structure of the enzyme, the nature of its active center, and the electrostatic conditions and physical-chemical state of the substrate. It was observed that changing the electrostatic conditions by adding, for example, charged phospholipids as phosphatidic acid into the film facilitates the penetration of the active center and hence enhances the enzymatic activity (Hirasawa et al., 1981; Geng et al., 1998; Chen and Barton, 1971).

In all experiments, a lag phase or slow hydrolysis was observed after the addition of the enzyme. The so-called lag-burst behavior is a general phenomenon described in detail for hydrolysis reactions catalyzed by PLA_2 (Verger et al., 1973; Apitz-Castro et al., 1982; Burack and Biltonen, 1994; Nielsen et al., 1999). In our experiments, the duration of the lag period depends to a large extent on the monolayer preparation and on the enzyme distribution in the subphase. Lipid packing defects, which are related to structural microheterogeneity, and composition heterogeneity have been

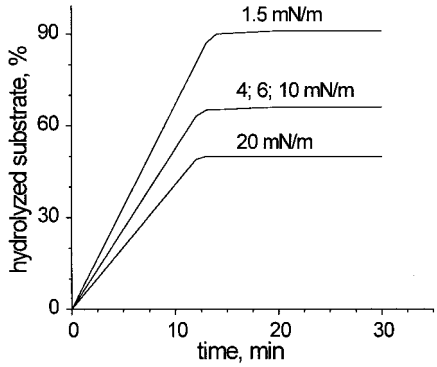

FIGURE 6 Yield of hydrolysis catalyzed by 255 units of PLD (*S. chromofuscus*) versus reaction time at different surface pressures (indicated). The subphase was an aqueous buffer solution with 50 mM $CaCl_2$, 150 mM NaCl, 10 mM Tris at pH 8.

correlated with the duration of the lag period (Hønger et al., 1996; Callisen and Talmon, 1998). The increase of the defect number decreases the duration of this period. Therefore, the lag phase is not shown in Fig. 6 and zero time corresponds to the beginning of the fast hydrolysis. During the lag phase the hydrolysis product, DPPA, accumulates and finally initiates the process of fast hydrolysis. However, the percentage of DPPC hydrolyzed during the lag phase must be below 5% and cannot be detected with high enough accuracy in the time-resolved scans at low surface pressure. In bilayer experiments it was demonstrated that the hydrolysis products segregate in the bilayer plane toward the end of the lag period (Burack et al., 1993; Jain et al., 1989; Nielsen et al., 1999). This segregation was caused by Ca^{2+} (Geng et al., 1998). At surface pressures below the plateau in DPPC/DPPA mixtures with small DPPA mole fraction, small domains can be already seen using Brewster angle microscopy. This indicates a phase separation in the monolayer, where DPPA-rich domains are surrounded by fluid DPPC. The presence of the lag phase is probably a result of a low enzyme-binding affinity to the substrate. It has been shown that PLD has a high affinity for DPPA-segregated domains (Stieglitz et al., 1999). This is not caused only by an electrostatic interaction between the positively charged docking region of PLD and the negatively charged DPPA because it was found that not all charged lipids induce PLD activity (Kanfer et al., 1996).

As one can see from Figs. 5 and 6 there is a certain DPPA concentration where the hydrolysis stops. This inhibiting DPPA concentration is a function of the monolayer pressure. For example, using 255 units of PLD, the hydrolysis stops at 20 mN/m after the production of 50% DPPA, whereas at 1.5 mN/m, 90% DPPA is necessary to inhibit the reaction. This inhibitory effect of DPPA at higher concentrations is believed to be partially caused by a surface pH shift. For negatively charged surfaces the pH value near the interface may be as much as 2 pH units smaller than the bulk pH. It was found (Qarles et al., 1969) that the apparent shift of the pH optimum of monolayer hydrolysis compared with the bulk pH and the inhibition effect of phosphatidic acid was considerably reduced if the H^+ ion as counterion was partially replaced by the Na^+ ion (Chen and Barton, 1971). The same quantity of DPPA, which inhibits PLD at higher pressure, is not capable of stopping the hydrolysis at low pressure (Fig. 6). This finding cannot be explained by a pH effect alone. It may also be connected with the miscibility behavior of substrate and hydrolysis product. The observed high-frequency shift of the symmetric and antisymmetric PO_2^- vibrations as well as of the carbonyl vibrations during the formation of DPPA indicates a reduced hydration or ion binding due to tighter headgroup packing. This can reduce the accessibility of the POC bond for an attack by PLD.

CONCLUSIONS

PM-IRRAS is a powerful technique to study the kinetics of hydrolysis processes catalyzed by phospholipases. The spectroscopic analysis of the phosphate groups provides a quantitative estimation of the fraction of substrate hydrolyzed. It was found that PLD exhibits maximum activity in the more disordered phase (LE) in contrast to PLA_2, which has its activity maximum in the two-phase region. The hydrolysis product DPPA can be considered as an important factor for the regulation of the catalytic process because this product remains in the monolayer and influences the monolayer structure and enzyme binding. A small amount of DPPA has to be accumulated for initiating the fast hydrolysis reactions. Higher concentrations of DPPA inhibit the hydrolysis. The critical inhibition concentration of DPPA is a function of the pressure in the monolayer. The inhibition effect of DPPA additionally depends on the surface pH and on the structural changes of the substrate induced by the hydrolysis product.

Helpful discussions with A. Gericke, University Halle, and B. Desbat, University Bordeaux, are gratefully acknowledged.
This work was supported by the Max Planck Society and the Deutsche Forschungsgemeinschaft (DFG).

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