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Monitoring of phospholipid monolayer hydrolysis by phospholipase A2 by use of polarization-modulated Fourier transform infrared spectroscopy

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Abstract

Polarization-modulated infrared reflection absorption spectroscopy (PM-IRRAS) was used to follow the hydrolysis of phospholipid monolayers at the air–water interface by phospholipase A2 (PLA2). The decrease in the intensity of the νC=O ester band of dipalmitoylphosphatidylcholine at 1733 cm^{-1} and the appearance of two new infrared bands in the 1530-1580 cm^{-1} region allowed to monitor phospholipid hydrolysis by PLA2. Indeed, the decrease in the intensity of the band at 1733 cm^{-1} was attributed to the enzymatic hydrolysis of the acyl ester linkage of the *sn*-2 fatty acid on the glycerol backbone whereas the doublet appearing at 1537 and 1575 cm^{-1} was attributed to the ν_a COO^- vibration of the newly formed calcium-palmitate. The presence of this band as a doublet indicates the formation of a crystalline-like calcium-palmitate monolayer. This observation supports our previously postulated mechanism for the formation of PLA2 domains at the air–water interface. Definitive assignment of the infrared bands has been possible by measuring PM-IRRAS spectra of the individual hydrolysis products (palmitic acid and lysopalmitoylphosphatidylcholine) as well as of 1-caproyl-2-palmitoyl-phosphatidylcholine and 1-palmitoyl-2-caproyl-phosphatidylcholine monolayers before and after hydrolysis by PLA2.

Keywords: Monolayer; Phospholipase A2; Infrared spectroscopy; Polarization-modulated infrared reflection absorption spectroscopy; Phospholipid hydrolysis

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1. Introduction

Phospholipase A2 (PLA2; EC 3.1.1.4) is an interfacially activated enzyme that catalyzes regio- and stereospecific hydrolysis of the *sn*-2 acyl ester linkage of *sn*-3-glycero-phospholipids [1], producing a fatty acid and a lysophospholipid. It is well recognized that PLA2 action towards organized lipid interfaces (vesicles, multilamellar dispersions and monolayers) is much higher than against isotropically dispersed phospholipids. The activity of this enzyme varies accordingly with the physico-chemical properties of the interface [2]. Several methods can be used to follow the activity of this enzyme on phospholipid monolayers (for a review, see Piéroni et al. [3]). These include the monitoring of molecular area, surface pressure and surface potential as a function of time [4] which allows to gain information on the kinetics of hydrolysis of phospholipid monolayers, the use of epifluorescence [5,6] and atomic force microscopy [7] which allow to observe the action of the enzyme on the micro- and nanoscopic scale, respectively, and the measurement of the effect of PLA2 adsorption on monolayer structure by grazing incidence X-ray diffraction [8]. Infrared reflection absorption spectroscopy (IRRAS) has also been used to follow this enzymatic hydrolysis process in situ at the air–water interface [9]. In the present study, polarization-modulated infrared reflection absorption spectroscopy (PM-IRRAS) was used to study the hydrolysis of dipalmitoylphosphatidylcholine (DPPC) monolayers by PLA2. In order to make a definite assignment of the infrared bands, we also measured the spectra of monolayers of pure PLA2 hydrolysis products (palmitic acid and lysopalmitoylphosphatidylcholine, C16Lyso-PC) as well as the spectra of asymmetric phospholipids bearing a short chain water-soluble fatty acid either at *sn*-1 or *sn*-2 position of the glycerol backbone in the presence and absence of PLA2.

Moreover, PM-IRRAS was used to get insight into the organization of the PLA2 domains observed after monolayer hydrolysis as well as to directly demonstrate the involvement of calcium in this process. We and co-workers have previously shown that hydrolysis of DPPC monolayers leads to the formation of PLA2 domains [5,10] that is driven by the presence of negatively charged fatty acid hydrolysis products [4,11] after their calcium-induced phase separation [12]. The remaining question concerned the direct demonstration of the involvement of calcium in this process. PM-IRRAS was thus also used to obtain this information.

2. Materials and methods

N. naja naja venom PLA2, palmitic acid, DPPC and C16Lyso-PC were purchased from Sigma and used without further purification. Asymmetric phospholipids 1-caproyl-2-palmitoyl-*sn*-3-phosphatidylcholine (C6C16-PC) and 1-palmitoyl-2-caproyl-*sn*-3-phosphatidylcholine (C16C6-PC) were kindly provided by Professor Mary Roberts (Boston College, Boston, USA). Their synthesis has been previously described in detail [13]. NaCl (Chempure, $>99.9\%$), $CaCl_2$ (Chempure, $>99.9\%$), Tris (hydroxymethyl) aminomethane (Sigma $>99.9\%$) were used as received. The water used was filtrated on a Millipore Nanopure System. Its resistivity and surface tension were higher than 18 MΩ cm and 71 mN m^{-1}, respectively.

PM-IRRAS spectra were recorded by co-addition of 300 scans using a Nicolet 740 spectrometer equipped with a HgCdTe detector cooled to 77 K. A detailed description of the PM-IRRAS set-up as well as the experimental procedure to acquire the spectra has been described previously [14]. Briefly, the infrared beam was polarized by a ZnSe grid and modulated by a ZnSe photoelastic modulator (PEM) between polarization (p) and (s) which are, respectively, in the plane and perpendicular to the plane of incidence. For all experiments, the PEM was set to give halfwave retardation at wavelength of 7 μm. The light beam was reflected at the air–water interface before being focused on the detector [15]. Optimal conditions for detection were obtained with an incidence angle of approximately 75° relative

to the normal at the water surface. The PM-IRRAS signal can be expressed [14] as follows:

$$S = C\frac{(R_p - R_s)J_2(\varphi_0)}{(R_p + R_s) + (R_p - R_s)J_0(\varphi_0)}$$

where R_p and R_s are the polarized reflectivities, J_0 and J_2 are the zero and second-order Bessel functions, φ_0 is the maximum dephasing given by the photoelastic modulator, and C is a constant which depends on the electronic device. Optically, in the mid-infrared range, water behaves as a dielectric substrate and, therefore, contributes to the PM-IRRAS signal. In order to extract the weak absorption bands of the film and to get rid of the dependence on Bessel functions, the spectrum of the monolayer is divided by the spectrum of the subphase buffer. In the normalized spectra, the direction of the bands vs. the baseline indicates the orientation of the transition moment and, hence, of the molecular groups at the air–water interface. Indeed, for an incidence angle of 75°, it has been determined [14] that an upward-oriented band indicates a transition moment occurring preferentially in the plane of the monolayer, whereas a downward-oriented band reveals an orientation preferentially perpendicular to the monolayer. For a given oscillator strength, bands associated with transition moments that are parallel to the surface are more intense than those perpendicular to the surface [16]. When the angle θ between the transition absorption moment and the surface normal is varied, absorption vanishes at $\theta = 38°$. This value is essentially determined by the refractive index ($n = 1.3$) of the water subphase at 1650 cm^{-1}.

Monolayers were prepared on a home-built trough (surface area = 14 cm^2). Surface pressure was measured by the Wilhelmy method using a filter paper. Phospholipid monolayers were spread by using hexane/ethanol (9:1) on a buffer subphase containing 5 mM $CaCl_2$, 100 mM NaCl, 10 mM Tris (pH 8.9) at a temperature of 25°C. The monolayer was then compressed to the desired surface pressure and PLA2 was injected into the subphase (PLA2 concentration = 0.4 units ml^{-1}). The surface pressure was maintained constant during hydrolysis.

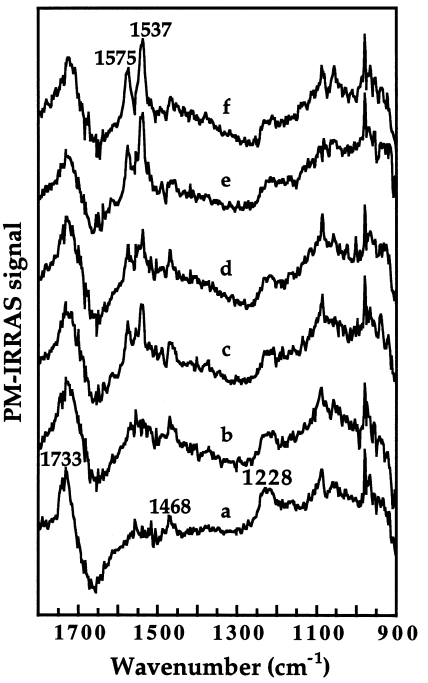

Fig. 1. PM-IRRAS spectra of DPPC monolayer hydrolysis by PLA2 (0.4 units ml^{-1}) at 8 mN m^{-1}. (a) Pure DPPC monolayer before and (b) 10 min, (c) 25 min, (d) 50 min, (e) 70 min, (f) 90 min after PLA2 injection into the subphase. Subphase contains 150 mM NaCl, 5 mM $CaCl_2$, 10 mM Tris (pH 8.9), $T = 25$°C.

3. Results and discussion

3.1. Evidence for monolayer hydrolysis by phospholipase A2

During the course of phospholipid monolayer hydrolysis by PLA2, a fatty acid and a lysophospholipid are produced. PM-IRRAS spectra of DPPC monolayer at the air–water interface in the 1800–900 cm^{-1} region measured at different extents of monolayer hydrolysis by PLA2 are shown in Fig. 1. The three most intense bands in the spectrum of DPPC before monolayer hydrolysis have been attributed to the νC=O ester

stretching band at 1733 cm^{-1}, the δCH_2 bending mode at 1468 cm^{-1}, and the $\nu_a P{=}O$ at 1228 cm^{-1} (spectrum a, Fig. 1). The assignment of these bands is in agreement with the data of [9]. The presence of the broad negative infrared band in the 1700–1640 cm^{-1} region has been previously documented [14,15,17,18]. In brief, the water absorption band is centered at approximately 1640 cm^{-1} which corresponds to the position where the PM-IRRAS spectrum changes abruptly showing a positive band followed immediately by a negative band. This behavior is due to an abrupt variation of the refractive index of the aqueous subphase in this range of frequency. Dluhy et al. [19] and Gericke et Hühnerfuss [20] have also interpreted the observation of this band by IRRAS in terms of an abrupt change of the refractive index of water. The fact that this band completely disappears when spectra are measured on D_2O [21] strongly argues in favor of this interpretation.

Upon monolayer hydrolysis by PLA2, a decrease in the intensity as well as a broadening of the C=O ester band at 1733 cm^{-1} is observed (spectra b–f, Fig. 1). The decrease of this band, which was also observed by Gericke and Hühnerfuss [9], is due to the hydrolysis of the *sn*-2 acyl ester linkage of DPPC by PLA2. The residual νC=O ester band at the end of the hydrolysis (spectrum f, Fig. 1) is due to the *sn*-1 acyl ester linkage of C16Lyso-PC hydrolysis product that remains at the interface. The broadening of this band can be interpreted by the formation of an increasingly disordered monolayer during hydrolysis [16]. Additional information comes from the use of the asymmetric phospholipid C16C6-PC. Indeed, hydrolysis of C16C6-PC by PLA2 produces a soluble fatty acid (caproic acid, C6) and an insoluble C16Lyso-PC. Fig. 2 shows the PM-IRRAS spectra before (spectrum a) and after (spectrum b) hydrolysis of the C16C6-PC monolayer by PLA2. The only difference between these two spectra is the decrease in the intensity of the νC=O ester stretching band at 1733 cm^{-1}. As in the case of DPPC monolayer hydrolysis, the decrease in the intensity of this band is due to the hydrolysis of the *sn*-2 acyl ester linkage by PLA2. The infrared spectrum of pure C16Lyso-PC is presented in Fig. 3. This spectrum is very similar to the one of pure DPPC before hydrolysis (compare with spectrum a, Fig. 1). Indeed, the observation of a νC=O ester band at 1732 cm^{-1}, a δCH_2 band at 1468 cm^{-1}, and a $\nu_a P{=}O$ band at 1228 cm^{-1} (see Fig. 3) supports our interpretation of the data.

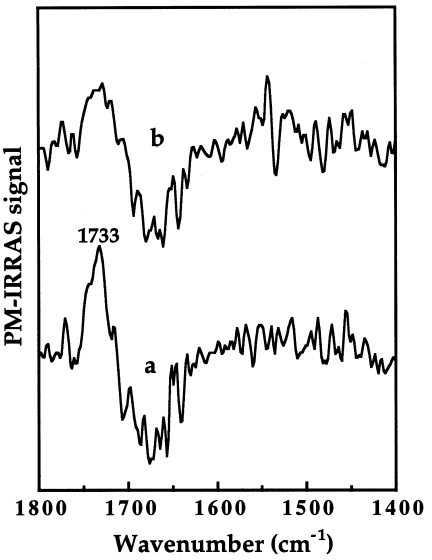

Fig. 2. PM-IRRAS spectra of C16C6-PC monolayer at 8 mN m^{-1}. (a) Pure C16C6-PC monolayer before and (b) 1 h after PLA2 injection (0.4 units ml^{-1}) into the subphase. Subphase is the same as in Fig. 1.

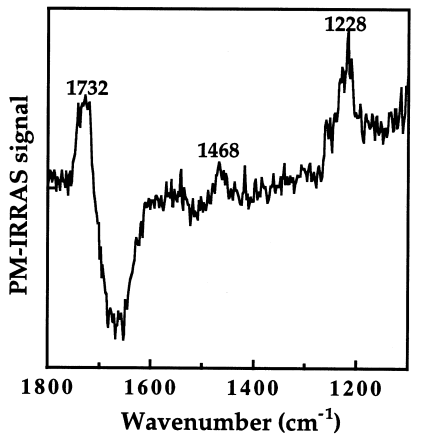

Fig. 3. PM-IRRAS spectrum of pure C16Lyso-PC monolayer at 8 mN m^{-1}. Subphase is the same as in Fig. 1.

Additional important changes in the spectra shown in Fig. 1 are observed upon monolayer hydrolysis by PLA2 (spectra b–f). Indeed, the appearance of two new bands at 1537 and 1575 cm^{-1} can be seen in Fig. 1 (spectra b–f). Gericke and Hühnerfuss [9] have also observed the appearance of new bands after PLA2 hydrolysis at 1542 cm^{-1} and a less intense band at 1578 cm^{-1} as well as an additional band at 1562 cm^{-1}. The bands at 1562 and 1542 cm^{-1} were attributed to the main antisymmetric carboxylate vibrations (to the formation of a calcium-palmitate complex) whereas the band at 1578 cm^{-1} was postulated to originate from the $\nu_a COO^-$ absorption of the β-carboxylate group of aspartic acid residues of PLA2. Since the bands at 1537, 1562 and 1575 cm^{-1} are not observed after monolayer hydrolysis of C16C6-PC by PLA2 which leads to the solubilization of caproic acid (C6) into the subphase (spectrum b, Fig. 2), it can be postulated that the presence of fatty acids is responsible for the observation of these bands and that none of them can be attributed to amino acid residues of PLA2. Moreover, the absence of these bands in the spectrum of C16Lyso-PC (Fig. 3) suggests that they can not be attributed to this hydrolysis product.

Given that the intensity of the PM-IRRAS bands depends on the orientation of molecular species, the decrease of the band at 1733 cm^{-1} and the appearance of the bands at 1537 and 1575 cm^{-1} could be due to orientational changes of these functional groups. However, literature data show that the orientation of these functional groups remains unchanged where the carbonyl lies parallel to the plane of the water surface and the axis C2 of the carboxylate is close to the normal of the surface [14–16,18,22,23]. Moreover, the carboxylate group has a quite intense symmetric vibration in addition to the antisymmetric vibration that is observed in the present work. If an orientational change of this functional group would take place in the course of the measurements, an obvious spectral modification should be observed in the 1500–1400 cm^{-1} spectral range. However, no such change is observed in the spectra. The changes in the spectra observed at 1733, 1537 and 1575 cm^{-1} can thus be only attributed to the action of PLA2 on DPPC monolayers.

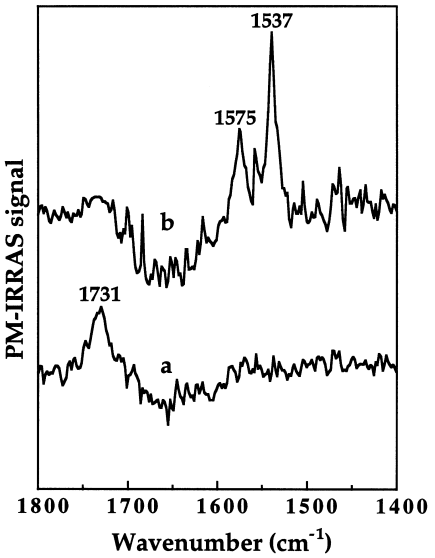

Fig. 4. PM-IRRAS spectra of C6C16-PC monolayer at 8 mN m^{-1}. (a) Pure C6C16-PC monolayer before and (b) 1 h after PLA2 injection (0.4 units ml^{-1}) into the subphase. Subphase is the same as in Fig. 1.

3.2. Formation of a calcium–palmitate complex upon monolayer hydrolysis

The use of the asymmetric phospholipid C6C16-PC is particularly useful for the assignment of the bands at 1537 and 1575 cm^{-1} because its hydrolysis by PLA2 produces an insoluble fatty acid (palmitic acid, C16) and a soluble C6Lyso-PC [4]. The assignment of the infrared bands can thus be directly confirmed in the presence of PLA2. Fig. 4 shows the PM-IRRAS spectra before and after complete hydrolysis of the C6C16-PC monolayer. Before PLA2 injection, a $\nu C{=}O$ ester stretching band at 1731 cm^{-1} can be seen in spectrum a (Fig. 4) as observed in the case of DPPC monolayer before hydrolysis (spectrum a, Fig. 1). At the end of hydrolysis (spectrum b, Fig. 4), this band disappears almost completely due to the solubilization into the subphase of the C6Lyso-PC species which contains the $\nu C{=}O$ ester groups. In addition, two new bands can be

seen at 1575 and 1537 cm^{-1} as well as a smaller component at 1562 cm^{-1} (spectrum b, Fig. 4). The position of the two bands at 1575 and 1537 cm^{-1} corresponds very well to the one of the doublet observed after DPPC monolayer hydrolysis (spectrum f, Fig. 1).

The precise assignment of these infrared bands has been achieved by measuring PM-IRRAS spectra of palmitic acid hydrolysis product. At alkaline pH, the carboxylic acid of fatty acids is known to be deprotonated and palmitate salts are readily formed. The results from Fig. 1 (spectrum f) and 4 (spectrum b) suggest that the doublet observed at 1537 and 1575 cm^{-1} after a large extent of DPPC hydrolysis by PLA2 can be assigned to the formation of an ionic salt of palmitic acid. However, in principle, these vibrations could either be assigned to a sodium- or a calcium-palmitate complex or both. Indeed, the subphase used contains 5 mM $CaCl_2$, 100 mM NaCl and 10 mM Tris at pH 8.9. In order to make a clear assignment of these bands observed after monolayer hydrolysis by PLA2, spectra of pure palmitic acid were measured on different subphases including each individual component of the buffer used for monolayer hydrolysis by PLA2 (see Figs. 5 and 6). In Fig. 5, the spectrum of palmitic acid on buffer (spectrum a) is compared with the one on a subphase containing only $CaCl_2$ (spectrum b). The spectra a and b in this figure show two well defined bands at 1538 and 1573 cm^{-1}, a small component at 1562 cm^{-1} and the δCH_2 vibration at 1468 cm^{-1}. There is a very good correlation between the position of these bands at 1538 and 1573 cm^{-1} (Fig. 5) and those observed at 1537 and 1575 cm^{-1} after a large extent of hydrolysis of DPPC (spectrum f, Fig. 1) and C6C16-PC (spectrum b, Fig. 4) by PLA2. The assignment of these bands to the formation of a calcium-palmitate complex is also supported by literature data. Indeed, Ahn and Franses [24] and Marshbanks et al. [25] have measured the infrared spectrum of LB films of stearic acid spread on a subphase containing 0.1 mM calcium (without

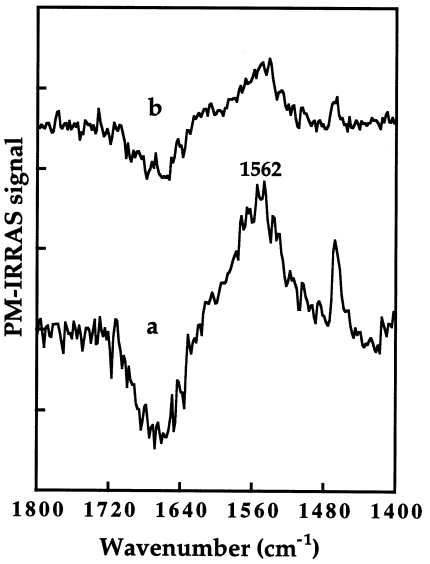

Fig. 6. PM-IRRAS spectra of pure palmitic acid monolayer spread on different subphases. Subphase contains (a) 150 mM NaCl and (b) 10 mM Tris (pH 8.9). Surface pressure is 8 mN m^{-1}.

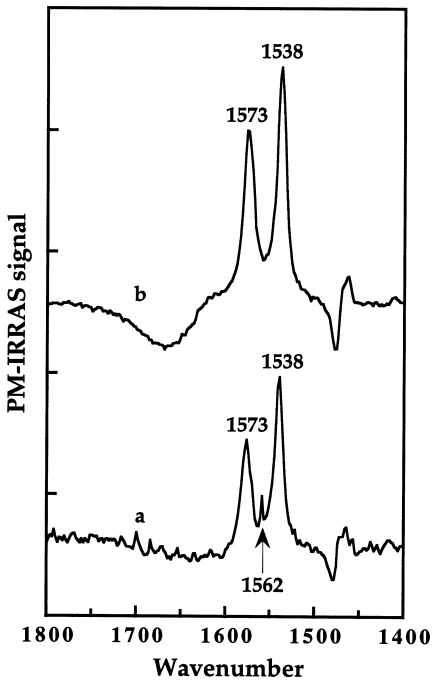

Fig. 5. PM-IRRAS spectra of pure palmitic acid monolayer spread on different subphases. (a) Subphase is the same as in Fig. 1 and surface pressure is 8 mN m^{-1}. (b) Subphase contains 5 mM $CaCl_2$ and surface pressure is 25 mN m^{-1}.

sodium) at pH 8 by ATR spectroscopy. Ahn and Franses [24] have observed a doublet in the 1540–1573 cm^{-1} region whereas Marshbanks et al. [25] have shown a spectrum with well defined bands at 1538 and 1574 cm^{-1}. These bands can thus be only attributed to the formation of a calcium–palmitate complex. This assignment is further supported by the spectrum of solid calcium-palmitate reported by Clarke [26] which also shows the presence of a doublet at 1540 and 1580 cm^{-1}. The two infrared bands at 1537 and 1575 cm^{-1} observed in Fig. 1 have thus been assigned to the ν_a COO^- vibration doublet of calcium–palmitate.

The spectra of palmitic acid on a subphase containing NaCl (spectrum a) or Tris buffer (spectrum b) are shown in Fig. 6. It can be seen that the two well-defined bands at 1538 and 1573 cm^{-1} observed in Fig. 1 (spectra b-f), 4 (spectrum b) and 5 are absent in these spectra and that, instead, a broad band located at 1562 cm^{-1} is observed for the sodium–palmitate complex. This broadening suggests a disordering of this monolayer in the presence of sodium. In addition, this assignment is supported by the observation by Clarke [26] of a single band at 1560 cm^{-1} in the infrared spectrum of solid sodium–palmitate.

3.3. The band at 1562 cm^{-1} is due to a structurally unstable complex

Gericke and Hühnerfuss [9] have attributed the band they observed at 1562 cm^{-1} to one of the main antisymmetric carboxylate vibrations of a calcium–palmitate complex. However, the data presented in Figs. 1–6 do not support this interpretation. The presence of such a band was not obvious in spectrum f of Fig. 1 although there are signs of this band in spectra c–e of Fig. 1 as well as in spectrum b of Fig. 5. Moreover, this band can be clearly observed in Fig. 4 (spectrum b) as well as in Fig. 5 (spectrum a). The following measurements have thus been performed to clarify the origin of this band which was intriguing. The monolayer of palmitic acid on a subphase containing only $CaCl_2$ was compressed to 5 mN m^{-1} and a spectrum was then quickly measured within 10 s using only 10 scans (spectrum a, Fig. 7). The position of the bands observed in this spectrum (spectrum a, Fig. 7) is the same as those shown in spectrum a of Fig. 5. The monolayer was then allowed to relax for 10 min and a new spectrum was recorded with 300 scans as usually performed (spectrum b, Fig. 7). The band at 1562 cm^{-1} in this spectrum has almost completely disappeared. The observation of this band at 1562 cm^{-1} when pure palmitic acid is spread onto a subphase containing only $CaCl_2$ (spectrum a, Fig. 7) as well the shape of the band of the sodium–palmitate complexes (spectrum a, Fig. 6) clearly demonstrates that this band cannot be due to the formation of a sodium–palmitate complex during the course of monolayer hydrolysis by PLA2. This band at 1562 cm^{-1} may thus be due to the formation of a structurally unstable complex as it vanishes almost completely in equilibrium conditions (spectrum b, Fig. 7). Such a com-

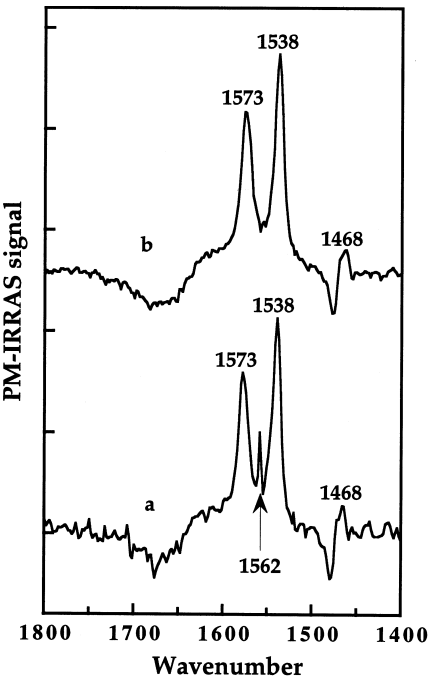

Fig. 7. PM-IRRAS spectra of pure palmitic acid monolayer spread on 5 mM $CaCl_2$. (a) Spectrum is measured immediately once surface pressure of 5 mN m^{-1} is reached with only 10 scans which requires 10 s acquisition time; (b) the film is allowed to relax for 10 min and a new spectrum is then measured (300 scans).

plex could be an amorphous calcium carboxylate complex which would exist until fatty acid domains are formed.

3.4. Involvement of calcium in the formation of phospholipase A2 domains

The presence of the doublet at 1537 and 1575 cm^{-1} has been interpreted by Clarke [26] as a crystal field effect which means that, in our conditions in monolayers at the air–water interface, calcium induces phase separation of palmitic acid hydrolysis products into highly organized two-dimensional crystal-like structures bearing an equivalent symmetry. This result is particularly interesting because it supports our postulated mechanism of the formation of PLA2 domains. Indeed, we and co-workers have previously proposed [5,10] that critical amounts of PLA2-produced fatty acids lead to monolayer lateral phase separation of negatively charged fatty acids. PLA2 would then adsorb onto these phase separated fatty acids and eventually form PLA2 domains. Furthermore, the use of the asymmetric phospholipids C16C6-PC and C6C16-PC allowed us to demonstrate that the presence of fatty acids is essential for the formation of PLA2 domains because these domains could be observed only when fatty acids were present at the air–water interface after monolayer hydrolysis, i.e. only in the presence of C6C16-PC [4]. Moreover, Maloney and Grainger [12] have shown that the presence of calcium is essential to the formation of both phase separated fatty acids and PLA2 domains. These authors have also demonstrated that phase separation occurs neither at low pH nor on calcium-free subphases. In the present work, we have observed infrared bands (the doublet at 1537 and 1575 cm^{-1}) that clearly show the involvement of calcium in the formation of phase separated crystalline-like domains of palmitate salts which represents the first step in the formation of PLA2 domains.

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