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Biochimica et Biophysica Acta 1560 (2002) 1–13

BIOCHIMICA ET BIOPHYSICA ACTA

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Behavior of a GPI-anchored protein in phospholipid monolayers at the air–water interface

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Received 22 May 2001; received in revised form 14 August 2001; accepted 27 August 2001

Abstract

The interaction between alkaline phosphatase (AP), a glycosylphosphatidylinositol (GPI)-anchored protein (AP-GPI), and phospholipids was monitored using Langmuir isotherms and PM-IRRAS spectroscopy. AP-GPI was injected under C16 phospholipid monolayers with either a neutral polar head (1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine monohydrate (DPPC)) or an anionic polar head (1,2-dipalmitoyl-*sn*-glycero-3-phospho-L-serine (DPPS)). The increase in molecular area due to the injection of protein depended on the surface pressure and the type of phospholipid. At all surface pressures, it was highest in the case of DPPS monolayers. The surface elasticity coefficient E, determined from the π–A diagrams, allowed to deduct that the AP-GPI–phospholipid mixtures presented a molecular arrangement less condensed than the corresponding pure phospholipid films. PM-IRRAS spectra suggested different protein–lipid interactions as a function of the nature of the lipids. AP-GPI modified the organization of the DPPS deuterated chains whereas AP-GPI affected only the polar group of DPPC at low surface pressure (8 mN/m). Different protein hydration layers between the DPPC and DPPS monolayers were suggested to explain these results. PM-IRRAS spectra of AP-GPI in the presence of lipids showed a shape similar to those collected for pure AP-GPI, indicating a similar orientation of AP-GPI in the presence or absence of phospholipids, where the active sites of the enzyme are turned outside of the membrane.

Keywords: Lipidic anchor; Alkaline phosphatase; PM-IRRAS; Langmuir film; Phospholipid monolayer

1. Introduction

Glycosylphosphatidylinositol (GPI)-anchored proteins are proteins localized on the outer layer of the plasma membrane [1,2]. They are clustered in membrane microdomains generally called rafts [3] in association with glycosphingolipids, sphingomyelin, cholesterol and others signaling proteins such as tyrosine kinases [4]. They are functionally diverse, including hydrolytic enzymes, protozoan surface proteins, adhesion protein, surface antigens, receptors and prion proteins.

Among the enzymes, which are encountered in membrane microdomains, alkaline phosphatase (AP; EC 3.1.3.1) is expressed in many cellular forms: bone, liver, kidney, intestine or placenta. It is a GPI-

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0005-2736/02/$ – see front matter
PII: S0005-2736(01)00405-9

anchored protein catalyzing non-specific hydrolysis of phosphate monoesters at alkaline pH in the presence of magnesium and zinc ions. The dimeric mammalian alkaline phosphatases are widely distributed enzymes. Each monomer has a GPI anchor.

To understand the organization of biological membranes and the interactions taking place between proteins and lipids, artificial membranes are very useful models. We investigated previously the incorporation of a GPI-anchored protein, alkaline phosphatase (AP-GPI), in biomimetic membrane systems such as liposomes [5,6]. However, as GPI proteins are localized in the outer layer of the cell membrane, monolayers at the air–water interface appear as a very suitable model to determine the nature of the lipid–protein interactions and the structure–function relations of such proteins [7,8].

Although many investigations on interactions of proteins with lipids using the Langmuir technique were reported [9–16], there are very few results concerning the nature of interactions involving GPI-anchored proteins with lipids [17].

We report here the monolayer organization of phospholipid–AP-GPI mixtures, monitored by compression isotherms and PM-IRRAS spectroscopy. Alkaline phosphatase prepared from bovine intestine was used as a model of a GPI protein to probe the interaction with membrane lipids.

Differential reflectivity measurements by polarization modulation of the incident light (PM-IRRA spectroscopy) proved to be an efficient method to obtain information on the structure and orientation of peptide or protein molecules in lipid monolayers at the air–water interface [18,19]. Recently Dziri et al. [12] characterized the effect of phospholipids on molecular organization and orientation of acetylcholinesterase without GPI anchor at the air–water interface. The enzyme was found to be fully inserted into the lipidic film during compression. A similar orientation of secondary structures of the enzyme was observed in the presence or absence of a lipidic film.

In a previous work, we described the orientation and protein–protein interactions of the AP-GPI monolayers at the air–water interface using thermodynamic and PM-IRRAS methods [20]. Based on the X-ray structure of human placenta alkaline phosphatase [21], an orientation of the protein with the great axis of the ellipsoid AP-GPI parallel to the interface has been suggested. In this work we will show that protein–lipid interactions depended on the nature of the lipids. AP-GPI modified the organization of the hydrocarbon chains of 1,2-dipalmitoyl-*sn*-glycero-3-phospho-L-serine (DPPS), while only the polar groups were affected in the case of 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine monohydrate (DPPC) at low surface pressure. The PM-IRRAS spectra of AP-GPI in the presence of lipids were similar to those of pure AP-GPI, indicating the same overall orientation of AP-GPI in the presence or absence of phospholipids. Based on this orientation, it was inferred that the active sites of the enzyme are turned outside of the membrane.

2. Materials and methods

2.1. Alkaline phosphatase

AP-GPI of bovine intestine was purified as described by Angrand et al. [5] modified by Ronzon et al. [22]. The enzyme was solubilized in Tris–HCl 10 mM, NaCl 150 mM, Mg^{2+} 1 mM, *n*-octyl β-D-glucopyranoside 2 mM, pH 8.5. The specific activity of the purified fraction was superior to 1000 U/mg. Alkaline phosphatase activity was measured according to the method of Cyboron and Wuthier [23]. It was performed at 37°C in 10 mM *p*-nitrophenyl phosphate (pNPP), 25 mM glycine and pH 10.5. The absorbance at 420 nm was monitored with a Kontron 932 spectrophotometer. One enzyme activity unit (U) was defined as the amount of enzyme able to catalyze the hydrolysis of 1 μmol of *p*-nitrophenyl phosphate per minute (pNPP/min) at 37°C ($\varepsilon_{420} = 18.5\ cm^{-1}\ mM^{-1}$).

The protein concentration was determined by the method of Bradford [24] modified by Read and Northcote [25], using bovine serum albumin as standard.

2.2. Other materials

The phospholipids, DPPC and DPPS, were obtained from Fluka and Sigma, respectively. The deuterated phospholipids, d_{62}-DPPS and d_{62}-DPPC, were purchased from Avanti Polar Lipids. All the phospholipids had a purity ≥99%.

Water (resistivity: 18.2 MW) was deionized and filtered with an ELGA apparatus (Lyon, France). All buffers prepared for the monolayer experiments were filtered onto filters of a 5 μm exclusion limit.

2.3. Langmuir setup

Films of alkaline phosphatase–phospholipids were obtained, in a nitrogen water saturated atmosphere, on a Teflon Langmuir trough (Riegler and Kirstein, Wiesbaden, Germany) thermostated at 21 ± 0.1°C. The trough ($6.2 \times 26.3 \times 0.5$ cm) was equipped with two symmetrical movable barriers controlled by an electronic device. The subphase was the usual buffer (TBS) of the protein: Tris–HCl 10 mM, NaCl 150 mM, Mg^{2+} 1 mM, pH 8.5. The surface pressure was measured by the Wilhelmy method, using a very thin plate of filter paper.

A Teflon Langmuir trough (surface 120 cm^2, subphase volume 83 cm^3) provided by Nima (Coventry, UK) was used for spectroscopic measurements.

2.4. Langmuir film formation

Phospholipids were dissolved in chloroform–methanol. The quantities of spread phospholipids were adjusted to obtain a surface pressure of 0 mN before starting the experiments. After 30 min incubation, the monolayers were compressed–decompressed before injection of AP-GPI under the phospholipid monolayers. Thirty minutes after injection of AP-GPI, the monolayer of protein–lipid was compressed and expanded. An additional amount of AP-GPI was injected and incubated for 30 min prior to the first compression–expansion cycle. Compression–expansion cycles were performed at a rate of 6 cm^2 min^{-1}. The isotherms were displayed by plotting the surface pressure versus the area per phospholipid molecule.

The measurement of the area increase due to the injection of AP-GPI for a given surface pressure π was determined from compression isotherms with two approximations. Firstly, the amount of AP-GPI incorporated into the phospholipid monolayers corresponded to the total amount of the added protein neglecting the fraction solubilized in the buffer subphase. This approximation seemed to be acceptable, considering the reversibility of the isotherms and the stability of the PM-IRRAS signals (see Section 3). Secondly, we assumed that the area occupied by a phospholipid molecule is not changed by the presence of the AP-GPI protein.

The apparent molecular area of the phospholipids was determined extrapolating graphically the π–A diagram to zero surface pressure from the linear part obtained at the end of the compression process.

The surface elasticity coefficient E, also named dynamic dilational modulus [26], was deduced from the π–A diagrams using the relation: $E = -A\, d\pi/dA$. As presented by Tomoaia-Cotisel et al. [27], this coefficient is used here to compare the stage of condensation of the different 2D states obtained with the well-known liquid expanded (L_1 type), liquid condensed (L_2 type) and solid states of the usual amphiphilic compounds, such as fatty acids or fatty alcohols.

2.5. PM-IRRAS measurements in situ

For spectroscopic measurements, deuterated chain (d-62) DPPS and DPPC were used. Langmuir films were prepared as indicated above.

At the air–buffer interface, mixed monolayers were monitored in situ by PM-IRRAS. The differential reflectivity measurements offer the possibility to follow changes in the secondary structure and orientation of the protein at the air–water interface. Spectra were recorded on a Nicolet 740 spectrometer equipped with a HgCdTe detector cooled at 77 K. A minimum of 400 scans were collected for each spectrum at a resolution of 8 cm^{-1}. The PM-IRRAS setup has been described elsewhere [18,28]. Briefly, the incident IR beam was polarized by a ZnSe polarizer and modulated by a ZnSe photoelastic modulator between parallel (p) and perpendicular (s) polarization to the incidence plane. The two-channel processing of the detected signal gives the differential reflectivity spectrum $\Delta R/R = (R_p - R_s)/(R_p + R_s)$ where R_p and R_s are the polarized reflectivities. To remove the contribution of the liquid water absorption, each IR spectrum is divided by the corresponding spectrum of the subphase. PM-IRRAS intensity is a function of the number of molecules per unit surface but also a function of the molecular conformation and orientation. During compression, the number of molecules per area unit increases, increasing the infrared absorption. Therefore PM-IRRAS spectra

were normalized by multiplying the infrared signal by the surface ratio before and after compression. After normalization the band intensity is only a function of the molecular orientation and molecular conformation. The optimal value of the angle of incidence for the detection was 75° relative to the interface normal. At this angle of incidence a negative band indicates a transition moment oriented preferentially perpendicular to the surface whereas a positive reflection absorption band indicates a transition moment oriented preferentially in the plane of the surface.

In order to take into account the surface increase, at constant pressure π, due to the protein insertion into a phospholipid monolayer, the difference spectrum (S) was computed according to the equation:

$$S = S_2 - (S_1 \times A_1 / A_2)$$

S_1 is the spectrum of pure phospholipid deposited on an area A_1 collected at a pressure p and S_2 the spectrum of mixed phospholipid–protein deposited on an area A_2, collected at the same pressure. For the same amount of phospholipids, A_2 is bigger than A_1 due to the insertion of the protein. The residual bands of water vapor were removed from the spectra S using an appropriate reference spectrum. The difference spectra were smoothed using a binomial function.

3. Results

3.1. Thermodynamic measurements

3.1.1. Isotherms of mixed phospholipid–protein monolayers

The compression isotherm of pure DPPS (Fig. 1A, curve b) on the TBS buffer showed a hysteresis more important than DPPC (Fig. 1A, curve a). The three expected steps were observed on the compression process of a pure DPPC monolayer: a liquid expanded state at low surface pressures, a phase transition corresponding to a mixture of liquid expanded and liquid condensed phases on the plateau region (5 mN/m) and a pure liquid condensed state at high surface pressures [29]. The apparent molecular areas were 72 and 59 Å^2/molecule for respectively pure DPPS and DPPC monolayers.

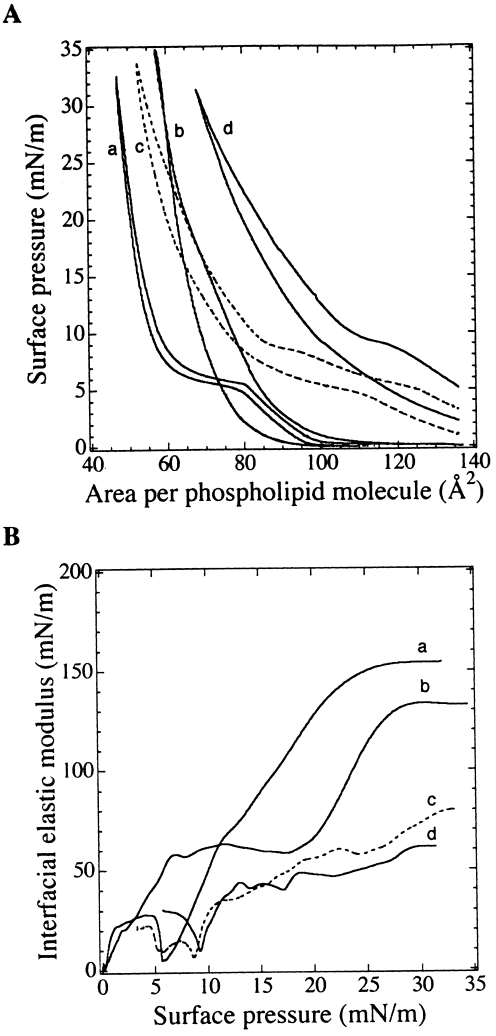

Fig. 1. (A) Surface pressure versus area per phospholipid molecule. Pure DPPC (a) and DPPS (b) were compared to isotherms recorded in the presence of 11.2 μg of AP-GPI for DPPC (c, dashed line) and 10.1 μg of AP-GPI for DPPS (d). (B) Interfacial elastic modulus versus surface pressure. The denominations for curves a–d are the same as in A.

The isotherm changes observed with the addition of a given amount of protein injected into the pure phospholipid monolayers are presented in Fig. 1A, curve c (mixture of DPPC and AP-GPI) and curve d (mixture of DPPS and AP-GPI). A transition due to the inserted protein occurred at a surface pressure in the range of 8–10 mN/m. At a given surface pressure, the injection of AP-GPI into the subphase introduced an important increase of the molecular area. This area is calculated for a phospholipid molecule either for pure phospholipid monolayers or for pro-

tein–phospholipid mixtures; thus, the increase represented directly the space occupied per phospholipid molecule due to the part of the AP-GPI molecules inserted into the lipid monolayer.

The increase of the molecular area induced by the injection of AP-GPI is plotted versus the AP-GPI amount injected at different surface pressures (DPPC in Fig. 2A and DPPS in Fig. 2B). From these plots, a linear increase of the area is observed for the phospholipids. In Table 1, we report the interfacial area occupied by the AP-GPI molecules in the different mixed protein–phospholipid monolayers. These areas have been calculated at a given surface pressure assuming both approximations presented in Section 2. At a given surface pressure, the values obtained for mixed films were less important than those for pure AP-GPI monolayers. Differences between the molecular areas obtained for AP-GPI in the DPPS and DPPC monolayers were also observed. Thus, at a surface pressure of 5 mN/m, the AP-GPI area was 132 nm^2 and 110 nm^2 for DPPS and DPPC films, respectively. This area decreased during compression to 26 nm^2 for the DPPS monolayer and to 17 nm^2 for the DPPC monolayer.

3.1.2. Elasticity surface

The interfacial elastic modulus (E) calculated from the compression isotherm diagrams, presented in Fig. 1A, provided an additional comparative parameter between the pure phospholipid and mixed protein–phospholipid monolayers.

The E values of pure DPPC (Fig. 1B, curve a) and DPPS (Fig. 1B, curve b) showed a plateau more or less important. For DPPC, the plateau is obtained at a surface pressure lower than 5 mN/m before a decrease corresponding to the liquid condensed–liquid expanded phase transition. A progressive increase in E is then observed at high surface pressure up to a maximum of 155 mN/m, corresponding to the liquid condensed state. In the case of pure DPPS (Fig. 1B, curve b), the liquid expanded state existed until 20 mN/m and the L_2 type condensed state appeared at surface pressures in the range of 25–35 mN/m.

In the presence of protein (Fig. 1B, curve c, mixture containing DPPC and curve d, mixture containing DPPS) the interfacial elastic modulus presented lower values than those observed for the pure phospholipid monolayers. The AP-GPI–phospholipid mixtures showed a molecular arrangement less condensed than the corresponding pure phospholipid films. The E values were similar to those obtained in the case of the liquid expanded state of the usual lipid monolayers. Furthermore, a minimum of E was observed for the film of DPPC containing protein (Fig. 1B, curve c) as well for the monolayer of DPPS containing protein (Fig. 1B, curve d), at surface pressures in the range of 8–10 mN/m. This minimum corresponded to the transition observed on the π–A compression isotherms (Fig. 1A).

3.2. PM-IRRAS measurements

3.2.1. Deuterated phospholipid monolayers

3.2.1.1. d_{62}-DPPS monolayer. Normalized PM-IRRAS spectra of pure d_{62}-DPPS film collected at different surface pressures are shown in Fig. 3A. The overall shape of these spectra did not change with the surface pressure but the band intensities increased. Some slight shifts were observed.

Table 1
Molecular areas of AP-GPI in mixed monolayers for different surface pressures

Surface pressure (mN/m)	AP-GPI in DPPC (nm^2/molecule)[a]	AP-GPI in DPPS (nm^2/molecule)[a]	Pure AP-GPI (nm^2/molecule)[b]
5	110	132	150
10	88	87	114
20	37	52	61
30	17	26	

[a]Molecular areas of AP-GPI in mixed films were calculated from the diagrams presented in Fig. 2. The slope was considered for lower quantities of protein.
[b]For pure AP-GPI, the areas were obtained from isotherms reported in a previous study [22]. A molecular mass for AP-GPI of 130 000 Da has been used for the calculations.

In the spectrum of d_{62}-DPPS measured at 2 mN/m surface pressure (Fig. 3A, spectrum a), the most intense band is negative relative to the baseline and is located near 1650 cm^{-1}. This is due to a different optical response of the covered and uncovered water surface and to spectral contribution of the water subphase [18,28]. The other bands appeared upward relative to the baseline, indicating that the projections of their transition dipole moments are preferentially oriented parallel to the surface plane [19]. This spectrum exhibited positive bands corresponding to the CD_2 symmetric and antisymmetric stretching vibrations at respectively 2095 and 2196 cm^{-1} [30]. The intense band located at 1728 cm^{-1} is assigned to the stretching of ester bonds. A broad band located between 1550 and 1610 cm^{-1} could be attributed to antisymmetric stretching vibrations of the COO^- group of the serine moiety [31,32]. However, Dluhy et al. [32] showed that the maximal intensity of this vibration occurs at 1623 cm^{-1} for phosphatidylserine multilayers in the presence or absence of Ca^{2+}, whereas Blaudez et al. [31] located the asymmetric carboxylate stretching bands at 1547 cm^{-1} in the case of cadmium arachidate. The ionic composition may influence the localization of this band. A broad band centered at 1240 cm^{-1}, could be assigned to antisymmetric P=O stretching vibrations. The CD_2 scissoring mode could be observed at 1088 cm^{-1}. The influence of the ionic composition of the subphase also affected these bands [19,33]. It was shown that interactions between divalent cations and phospholipid phosphate lead to an increase in the wave number of the antisymmetric P=O stretching vibration due to dehydration of the phosphate group [34].

The phospholipid film was compressed at 8 mN/m before collecting spectrum b in Fig. 3A. The intensities of the CD_2 bands increased by a factor 2 as compared with the intensities of the CD_2 bands in the spectrum of the sample obtained at 2 mN/m (Fig. 3A, spectrum a). The CD_2 symmetric/CD_2 antisymmetric stretching vibration ratio of 1.6 is conserved. The increase in the intensity is not due to an increase in the number of molecules under the infrared beam, since the spectra were normalized to obtain the same amount of molecules per unit surface. These intensity variations may be caused to changes in molecular orientation [34]. Besides the increase in the peak intensities of CD_2, a shift is also observed. In fact CD_2 symmetric and antisymmetric stretching vibrations shifted from 2095 to 2089 cm^{-1} and from 2196 to 2193 cm^{-1}, respectively. Their high intensities and their shift to lower wave numbers indicated a higher organization and a more rigid structure of the deuterated chains at the interface caused by the compression.

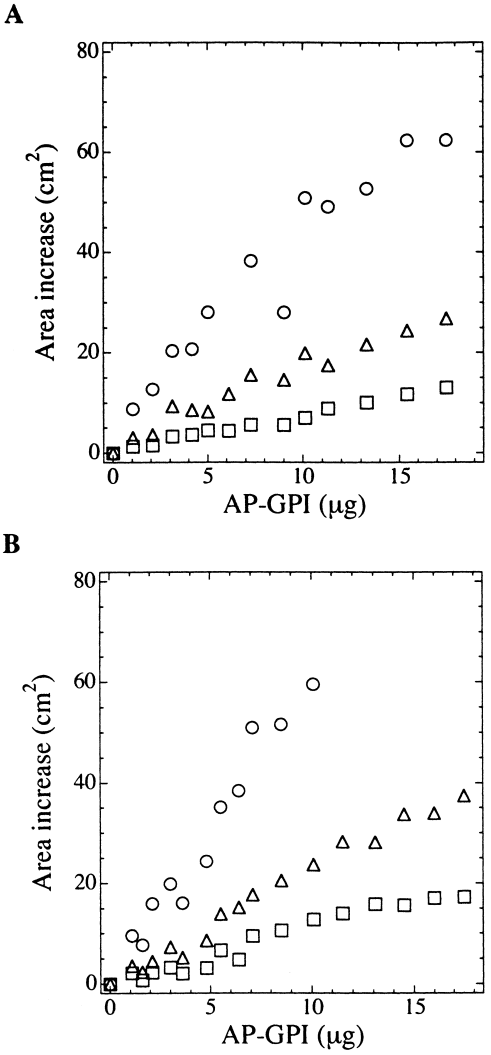

Fig. 2. Surface increase due to protein insertion into the lipid monolayer as a function of the amount of AP-GPI injected at a given surface pressure: ○, 5 mN/m; △, 20 mN/m; □, 30 mN/m. Lipid monolayer: (A) DPPC; (B) DPPS.

Compression of the phospholipid film to higher surface pressures (15 and 30 mN/m, spectrum c and d, respectively, in Fig. 3A), led to an increase in CD_2 band intensities without any shift. At 30 mN/m, an intensity increase and a shift of the ester bonds from 1733 cm^{-1} to 1739 cm^{-1} were observed. This shift

indicated hydrogen bonded ester groups with the water molecules at low surface pressure, and dehydration of these groups at high surface pressure. The shapes and positions of the other bands remained almost unchanged during the compression.

3.2.1.2. d_{62}-DPPC monolayer. Normalized PM-IRRAS spectra of the d_{62}-DPPC monolayer collected under the same conditions as for d_{62}-DPPS are shown in Fig. 3B (spectra a–d).

At the lowest surface pressure, the CD_2 symmetric and antisymmetric stretching vibrations are centered at 2096 and 2195 cm^{-1}, respectively (Fig. 3B, spectrum a). At higher pressures the same modifications as for d_{62}-DPPS were observed, presumably reflecting an increase of the conformational order of the deuterated chains (Fig. 3B, spectra b–d).

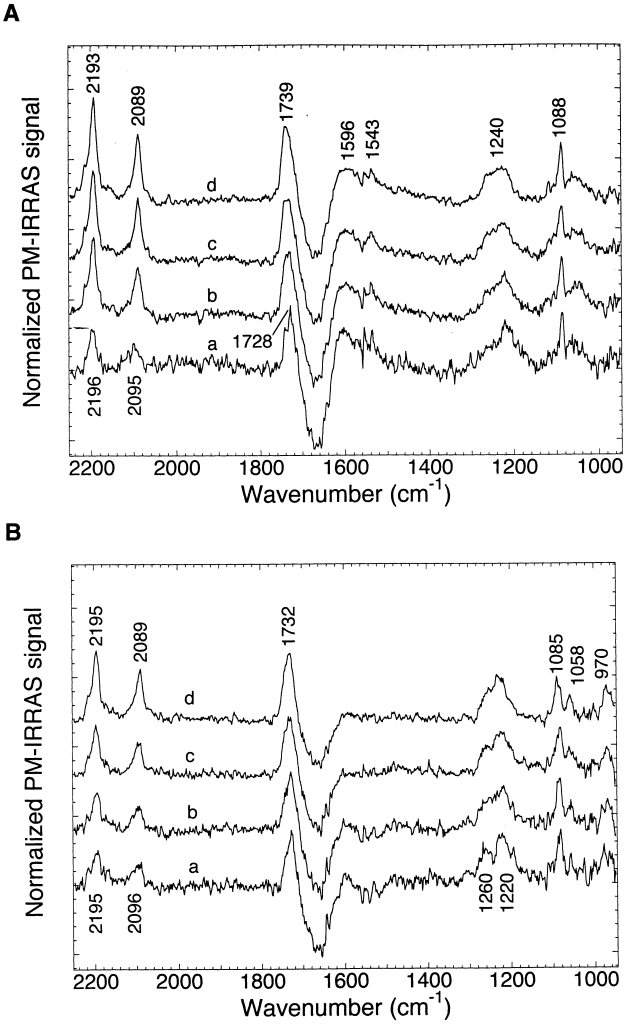

Fig. 3. Normalized PM-IRRAS spectra collected at different surface pressures for pure phospholipids. Spectra were measured at 2 mN/m (a), 8 mN/m (b), 15 mN/m (c) and 30 mN/m (d). (A) d_{62}-DPPS. (B) d_{62}-DPPC.

Comparison in the CD_2 region of the spectra of d_{62}-DPPC (Fig. 3B, spectrum b) and d_{62}-DPPS (Fig. 3A, spectrum b), both measured at 8 mN/m, indicated that the DPPS monolayers were more ordered than the DPPC monolayers. This is in agreement with *E* values and with previous results [34].

The band located at 1728 cm^{-1} (Fig. 3B, spectrum a), assigned to the stretching of ester bonds, shifted to 1732 cm^{-1} when the film was submitted to 30 mN/m (Fig. 3B, spectrum d). The broad 1240 cm^{-1} band, assigned to antisymmetric P = O stretching vibration, was composed of two components located at 1220 and 1260 cm^{-1}, suggesting that a part of the phospholipid molecules interact with magnesium ions present in the buffer. The choline residue of DPPC is evidenced by the presence of C-O-P stretching vibrations at 1058 cm^{-1} and of asymmetric stretching modes of the trimethylammonium group located at 970 cm^{-1} [12,35].

3.2.2. AP-GPI–deuterated phospholipid monolayers

3.2.2.1. Mixed films AP-GPI–d_{62}-DPPS. Normalized PM-IRRAS spectra of the mixed film d_{62}-DPPS–AP-GPI collected at different surface pressures are shown in Fig. 4A, and the difference spectra, calculated as described in Section 2, are displayed in Fig. 4B. Deuterated phospholipids were used to distinguish the C-D vibrational modes of phospholipids from the C-H vibrational modes of protein.

At a surface pressure of 2 mN/m (Fig. 4A, spectrum a), addition of AP-GPI led to decreasing intensities of the phospholipid bands in comparison with the spectrum of the d_{62}-DPPS monolayer at the same surface pressure (Fig. 3A, spectrum a). The decrease in intensity is predominantly due to a decrease in the number of phospholipid molecules under the infrared beam. To better identify group moieties of phospholipids affected by the protein, we determined difference spectra by taking into account the decrease in the number of phospholipid molecules (Fig. 4B). At 2 mN/m, the phospholipid groups were not affected by the presence of AP-GPI as shown by a relatively

flat baseline at characteristic wave numbers of deuterated phospholipids (Fig. 4B, spectrum a′). The region between 1600 and 1700 cm^{-1} showed an increase in the intensity of the PM-IRRAS signal in the spectrum of the AP-GPI–d_{62}-DPPS mixture (Fig. 4A, spectrum a). This is clearly seen with the corresponding difference spectrum (Fig. 4B, spectrum a′). Penetration of AP-GPI into the d_{62}-DPPS monolayer led to the appearance of amide I and amide II bands located at 1640 and 1540 cm^{-1}, respectively. The ratio of maximum intensities of amide I and amide II bands is 1.2. According to the usual assignment [36], the 1633 cm^{-1} component band was attributed to β-sheet structures while the weak 1647 cm^{-1} shoulder was tentatively assigned to α-helix structures. Usually, the α-helix component is located at a higher wave number (1653–1658 cm^{-1}). However, from simulated and PM-IRRAS spectra it was observed that a variation of the tilt angle between the helical axis and the normal of the plane could lead to a shift of the band in the PM-IRRAS spectra [19]. If the α-helix is perpendicular to the interface (tilt angle 0°) the absorption band is at 1656 cm^{-1}, while for an α-helix parallel to the interface (tilt angle 90°) the band is at 1649 cm^{-1}.

The spectrum of the mixture of d_{62}-DPPS and AP-GPI collected at 8 mN/m (Fig. 4A, spectrum b) showed a decrease in the intensities of CD_2 and ester bands as compared with the spectrum of pure phospholipid measured at the same surface pressure (Fig. 3A, spectrum b). This decrease was better seen in the difference spectrum (Fig. 4B, spectrum b′) where three negative bands appeared at 2093, 2196 and 1735 cm^{-1}. This suggests that the deuterated chain of phospholipids was more disordered in the presence of protein. Phosphate groups were not affected, as evidenced by the flat baseline between 1000 and 1300 cm^{-1} (Fig. 4B, spectrum b′). The intensities of amide I and amide II bands increased in comparison with the difference spectrum at 2 mN/m (Fig. 4B, spectrum a′), but the amide I/amide II ratio was always 1.2.

At higher surface pressures (15 mN/m and 30 mN/m), the shape of the normalized PM-IRRAS spectra of the mixed films (Fig. 4A, spectra c and d) remained unchanged in comparison with spectrum b in Fig. 4A. The intensities of the bands associated to deuterated phospholipids in the protein–lipid mixture were always weaker than those of a pure phospholipid film at the same surface pressure. CD_2 symmetric and antisymmetric stretching vibrations shifted with increasing pressure from 2093 to 2089 cm^{-1} and from 2196 to 2193 cm^{-1}, respectively. The corresponding difference spectra (spectra c′ and d′, Fig. 4B) showed a slight increase in the intensities of amide I and II bands during the compression. Their positions as well as their intensity ratio were always the same, suggesting that no changes occurred in the secondary structure or orientation

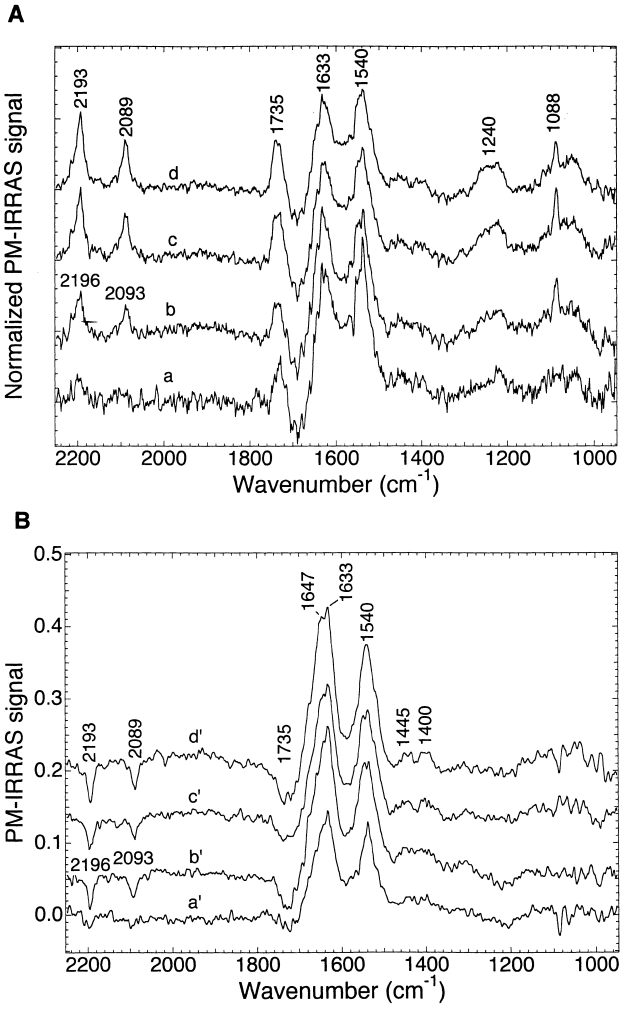

Fig. 4. Spectroscopic data of AP-GPI–d_{62}-DPPS mixed films collected at different surface pressures. The amount of AP-GPI injected into the subphase was 11.8 μg. Spectra were measured at 2 mN/m (a,a′), 8 mN/m (b,b′), 15 mN/m (c,c′) and 30 mN/m (d,d′). (A) Normalized PM-IRRAS spectra of d_{62}-DPPS–AP-GPI. (B) The same spectra after subtraction of the corrected lipid spectrum (see Section 2).

of the AP-GPI during compression. The broad peaks located at 1445 and 1400 cm^{-1} could originate from CH_2 and CH_3 bands of the protein.

3.2.2.2. Mixed films AP-GPI–d_{62}-DPPC. Normalized PM-IRRAS spectra of the mixed film d_{62}-DPPC–AP-GPI collected at different surface pressures are shown in Fig. 5A, and the difference spectra, calculated as described in Section 2, are displayed in Fig. 5B.

At a surface pressure of 2 mN/m, the PM-IRRAS spectrum of the mixture (Fig. 5A, spectrum a) showed no change as compared with the PM-IRRAS spectrum of pure d_{62}-DPPC (Fig. 3B, spectrum a). This was confirmed by the corresponding difference spectrum (Fig. 5B, spectrum a′). It is noisy without visible amide bands in contrast to what was observed in the case of d_{62}-DPPS (Fig. 4B, spectrum a′). The characteristic groups of phospholipids were not affected by the presence of protein at this surface pressure. However, an increase in area was observed on the isotherm presented in Fig. 1A.

The d_{62}-DPPC–AP-GPI spectra collected at 8 mN/m and 15 mN/m (Fig. 5A, spectra b and c) showed some modifications in comparison with the corresponding spectra of d_{62}-DPPC (Fig. 3B, spectra b and c). The band intensity at 1731 cm^{-1} is lower in the spectrum of mixed films than in the spectrum of the pure phospholipid. The difference spectra exhibited a negative band located at 1731 cm^{-1}, corresponding to the ester carbonyl group (Fig. 5B, spectra b′ and c′). Only the ester group of d_{62}-DPPC was disturbed by the protein since no aliphatic chain changes appeared between 2000 and 2200 cm^{-1}. A broad band appeared at 1540 cm^{-1}, corresponding to the amide II band. The amide I band appeared at 1638 cm^{-1} on the difference spectra (Fig. 5B, spectra b′ and c′). The presence of the amide I and II bands confirmed the incorporation of AP-GPI into the monolayer.

The d_{62}-DPPC–AP-GPI spectrum collected at a higher surface pressure (30 mN/m; spectrum d, Fig. 5A) indicated that the signal/noise ratio increased. A shift of the two CD_2 symmetric and antisymmetric stretching vibrations from 2096 to 2089 cm^{-1} and from 2197 to 2195 cm^{-1} with a slight increase in their intensities was observed. The corresponding difference spectrum (Fig. 5B, spectrum d′) exhibited five negative phospholipid bands located at 2197, 2089, 1731, 1224 and 1089 cm^{-1}. Therefore the AP-GPI disturbed strongly the organization of the DPPC molecules at high surface pressures. The intensities of the amide I and amide II bands increased by a factor of approx. 2 as compared with spectrum c′, without affecting the shapes and intensity ratio of these bands. This suggests that the intensity increase was not due to a change in the orientation of the AP-GPI but to an increase in the quantity of protein molecules under the infrared light spot.

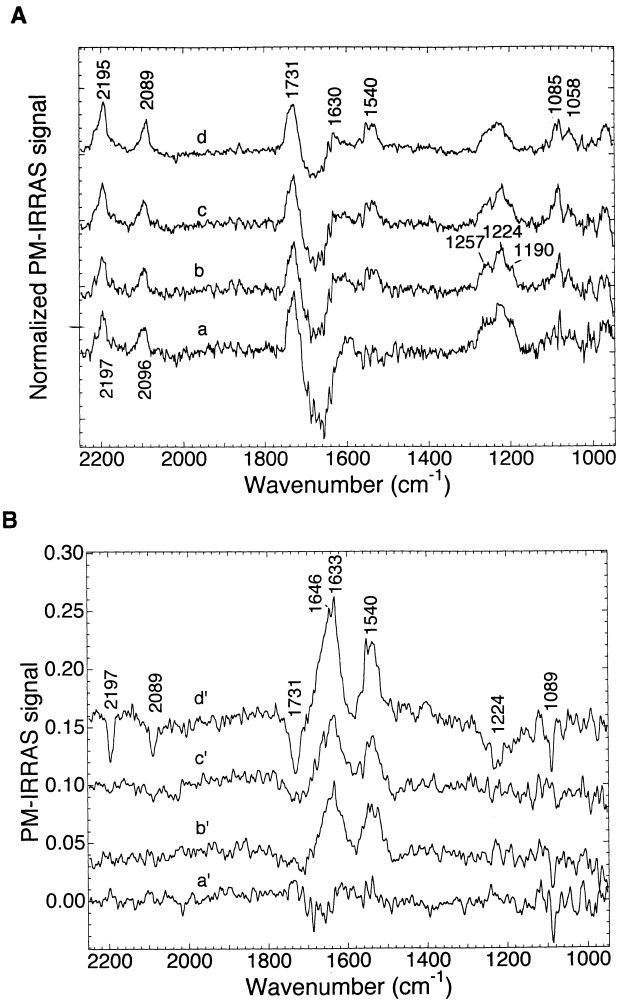

Fig. 5. Spectroscopic data of AP-GPI–d_{62}-DPPC mixed films collected at different surface pressures. The amount of AP-GPI injected into the subphase was 11.8 μg. Spectra were measured at 2 mN/m (a,a′), 8 mN/m (b,b′), 15 mN/m (c,c′) and 30 mN/m (d,d′). (A) Normalized PM-IRRAS spectra of d_{62}-DPPC–AP-GPI. (B) The same spectra after subtraction of the corrected lipid spectrum (see Section 2).

3.2.2.3. Comparison between the spectra of AP-GPI in the absence and presence of lipid monolayers. Overnight compression of the d_{62}-DPPS–AP-GPI mixture at 30 mN/m led to the difference spectrum of AP-GPI presented in Fig. 6 (full line). This surface pressure was believed to coincide with the packing density of biological membranes or lipid bilayers [37–39]. The amide I band exhibited two strong absorptions at 1655 cm^{-1} and 1638 cm^{-1}. The comparison between the other difference spectra (Fig. 4B) and this spectrum showed a shift of the component band from 1647–1648 cm^{-1} to 1655 cm^{-1} as well as a shift of the other component band from 1633 cm^{-1} to 1638 cm^{-1}. The intensity of the higher wave number band is greater than the intensity of the lower wave number band. The amide II band is located at 1540 cm^{-1}. The amide I/amide II area ratio is about 1.

The PM-IRRAS spectrum of AP-GPI alone collected after overnight compression is presented in Fig. 6 (dotted line). The spectra upon overnight compression are similar except for the position of the band located at 1633 cm^{-1} (AP-GPI alone) and 1638 cm^{-1} (mixed film). The amide I/amide II intensity ratio of 1.2 observed on the spectrum collected for the AP-GPI monolayer was slightly different from the ratio observed for the mixture.

In addition, we observed increasing intensities of the protein bands located at 1450 and 1403 cm^{-1}, assigned respectively to C-H scissoring (CH_2 and CH_3) and other C-H vibrations such as wagging vibrations or isopropyl symmetric bending [30], as compared with those observed with DPPS–AP-GPI mixtures.

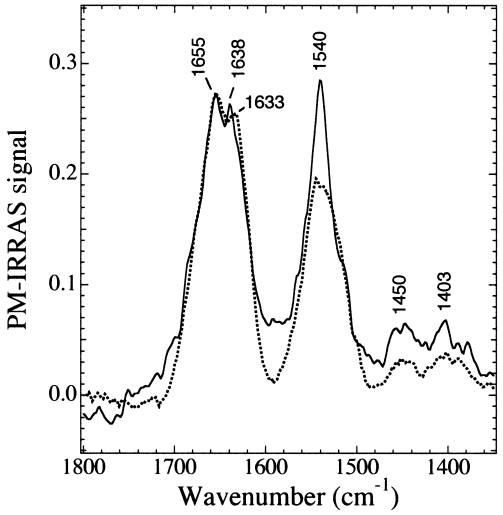

Fig. 6. Comparison of PM-IRRAS spectra of AP-GPI. Difference spectra of d_{62}-DPPS–AP-GPI (———) and AP-GPI alone (···) after overnight compression at constant high surface pressure.

4. Discussion

The interaction of AP-GPI with phospholipid monolayers at the air–buffer interface was investigated through Langmuir compression–decompression isotherms and PM-IRRAS spectroscopy. The phospholipids used present the same alkyl saturated chains (C_{16}) coupled to a negative (DPPS) or zwitterionic (DPPC) polar head group. These lipids are commonly used for the characterization of lipid–protein interactions [40,41].

4.1. Molecular area of AP-GPI in the mixed film

The increase in molecular area in the presence of AP-GPI at a given surface pressure clearly indicates the insertion of the protein into the different phospholipid films (Fig. 1A). Assuming a molecular mass of 130 kDa for the AP-GPI dimer, up to 100 pmoles of protein were injected into the subphase. For example at 5 mN/m, a total area increase of 60 cm^2 on a DPPC monolayer (Fig. 2A) corresponds to approx. 10^4 protein molecules per mm^2, considering the incorporation of the total amount of AP-GPI injected. Kloboucek et al. [17] used the same assumption to describe the insertion of a lipid-anchored glycoprotein in a DMPC monolayer. The linear relationship between the increase in area and the amount of protein injected (Fig. 2) is an additional argument for the interfacial localization of the protein. The calculated molecular areas of AP-GPI in the presence of lipids (Table 1) are lower than those determined in the case of pure AP-GPI monolayers. Consequently, this suggests that the mixed monolayer components interacted in such a way that they changed the intrinsic value of area per molecule of the AP-GPI and/or the phospholipids. In fact, the molecular areas of AP-GPI were calculated assuming that the molecular areas of phospholipids were not affected by the presence of protein at the same surface pressure.

4.2. AP-GPI–phospholipid interactions

The surface pressure–area isotherms (Fig. 1A, curves c and d) as well as the interfacial elasticity modulus (Fig. 1B, curves c and d) indicated a sort of transition around 8–10 mN/m during the compression step. A similar behavior was observed in the case of other proteins or peptides [42]. Trommeshauer and Galla [43] proposed three possible explanations for this transition: (i) the protein undergoes a conformational change, (ii) the lateral arrangement or orientation of the protein within the lipid monolayer is altered, (iii) the protein or the protein–lipid aggregates are squeezed out of the surface monolayer into the subphase. This last hypothesis is not consistent with our results since a similar isotherm is obtained after a second compression following an expansion (data not shown). Moreover, the intensity of the PM-IRRAS spectra of the protein did not decrease during compression, and overnight compression of the mixed monolayer did not produce a squeezing out of the protein into the subphase (Fig. 6). The possibility of a reorientation or a conformational change of the protein during compression is not in agreement with the PM-IRRAS signal. The shapes and intensity ratio of the amide I/amide II bands were relatively similar on the difference spectra during compression, suggesting the same orientation irrespective of the surface pressure values or the nature of the phospholipid used. In the study of Cornut et al. [19], the amide I/amide II ratio varies drastically from 6.5 to −1, with changes of orientation of the α-helix from parallel to perpendicular at the air–water interface.

Addition of AP-GPI to either d_{62}-DPPS or d_{62}-DPPC at 2 mN/m did not affect the deuterated phospholipid groups as probed by PM-IRRAS. At a higher surface pressure (8 mN/m), an increase in the lipid ordering of mixture AP-GPI–d_{62}-DPPS appeared, as evidenced by the changes in the PM-IRRAS spectra around 2196, 2093 and 1735 cm^{-1} (Fig. 4). In contrast, the infrared difference spectra of the d_{62}-DPPC–AP-GPI mixture collected at 8 mN/m or 15 mN/m showed only a perturbation of the ester groups of d_{62}-DPPC due to the presence of the protein (Fig. 5).

The interaction of AP-GPI with d_{62}-DPPS must be different from that with d_{62}-DPPC. In the latter case, the bands corresponding to the deuterated acyl chains were not modified at a surface pressure of 8–15 mN/m. The intensities of the infrared difference spectra associated to protein in the presence of d_{62}-DPPS were always higher than in the presence of d_{62}-DPPC, irrespective of the surface pressure (Figs. 4B and 5B). Moreover, the signal/noise ratio is greater for mixed AP-GPI–d_{62}-DPPS than for AP-GPI–d_{62}-DPPC. Since the same quantity of AP-GPI was used, it is possible that the environment of the protein is different between the two cases. It is possible that a lower quantity of AP-GPI would be incorporated into d_{62}-DPPC monolayers. However, the surface increases for DPPC and DPPS (Fig. 2) were of the same order. The possibility of a heterogeneous distribution of the protein forming domains into the DPPC monolayer cannot be excluded. These differences in spectra intensities may originate from different protein hydration layers induced by the presence of DPPC or DPPS. We showed that an increase in the hydration of the protein leads to a lower PM-IRRAS signal due to the optical constants of water molecules [20]. AP-GPI could be more hydrated in the presence of d_{62}-DPPC monolayers than in the presence of d_{62}-DPPS.

This is an important observation since it infers that the surrounding charges of phospholipids could be a key factor in the insertion of GPI anchor protein into lipids. AP-GPI affected the hydrocarbon chains of d_{62}-DPPC only at higher surface pressure (30 mN/m) (Fig. 5). Such a difference has been reported recently for a peptide inserted into monolayers [44]. The authors showed that a penetrating peptide induced a low chain disorder in a neutral DPPC monolayer and strongly disordered the whole lipid structure of a 10% DPPS charged monolayer. In the same way Liu et al. [41] reported that neutral lipids such as DPPC incorporated into the erythrocyte membrane did not affect the interaction with the complement proteins while incorporation of anionic lipids such as DPPS increased the hemolysis.

4.3. Structure and orientation of AP-GPI in the mixed film

Recently the 3D structure of a mammalian dimeric alkaline phosphatase prepared from the human placenta (PLAP) was described [21]. The PLAP presents

about 90% sequence similarity with the AP-GPI used in this work and is supposed to present the same organization. Each monomer shows a typical α/β topology with 26.12% α-helix and 13.84% β-sheet. The general shape of the protein could be assimilated to a prolate ellipsoid with the α-helix structures oriented at approx. 45° with respect to the great axis.

The spectra of mixed monolayers of AP-GPI and phospholipids (Figs. 4B and 5B) were similar to those obtained for pure protein [20]. This suggests an identical orientation of the protein in the presence or absence of lipid. Therefore the protein is anchored at the interface; while the GPI anchor is standing up, the great axis of the ellipsoid model of AP-GPI becomes parallel to the interface. In natural membranes, this orientation allows the AP-GPI active site to be exposed to the extracellular medium.

Localization of the α-helix band around 1647 cm^{-1} (Figs. 4B and 5B) was observed for pure AP-GPI. None of the difference spectra (Fig. 4B and 5B) obtained presented an α-helix band located at 1655 cm^{-1}, except for the spectrum collected after overnight compression at 30 mN/m (Fig. 6). A similar spectrum was reported for the pure AP-GPI after overnight compression. However, in the case of mixed films, a shift of the component band from 1633 cm^{-1} to 1638 cm^{-1} is observed (Fig. 6). This shift could be due to an interaction of β-sheet domains with DPPS molecules or a modified orientation of the β-sheet structures affecting the intensity of the 1633 cm^{-1} component band. These changes observed in PM-IRRAS spectra are not characteristic of a denaturation process. In the case of denaturation, the areas of amide I and amide II decrease drastically, as reported for others proteins [45]. Moreover, the appearance of a β-sheet structure at 1626 cm^{-1} characteristic of denatured AP-GPI was not observed [20].

In conclusion, we built a membrane model including phospholipids with the same chain lengths and a neutral polar head (DPPC) or an anionic polar head (DPPS). Injecting the AP-GPI under the lipid monolayers, our results indicated an association of AP-GPI with all the phospholipids used. The PM-IRRAS data suggested a different interaction of the protein with DPPS and DPPC. At lower surface pressure, AP-GPI induced a strong disorder of the hydrophobic chains in the DPPS monolayer in contrast to the DPPC monolayer, where only the polar ester group is disturbed. Moreover, the shape of the spectra of AP-GPI are similar to those previously collected for pure AP-GPI monolayers, suggesting an orientation identical in the presence and absence of the lipid components. In this orientation the active sites are turned outside of the membrane in contact with the external buffer.

Acknowledgements

We thank Prof. René Buchet for helpful discussions. This work was supported by a grant from the CNRS (Physique and Chimie du Vivant).

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