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In Situ Study by Polarization Modulated Fourier Transform Infrared Spectroscopy of the Structure and Orientation of Lipids and Amphipathic Peptides at the Air-Water Interface

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ABSTRACT Free amphipathic peptides and peptides bound to dimyristoylphosphatidylcholine (DMPC) were studied directly at the air/water interface using polarization modulation infrared reflection absorption spectroscopy (PMIRRAS). Such differential reflectivity measurements proved to be a sensitive and efficient technique to investigate in situ the respective conformations and orientations of lipid and peptide molecules in pure and mixed films. Data obtained for melittin, a natural hemolytic peptide, are compared to those of $L_{15}K_7$, an ideally amphipathic synthetic peptide constituted by only apolar Leu and polar Lys residues. For pure peptidic films, the intensity, shape, and position of the amide I and II bands indicate that the $L_{15}K_7$ peptide adopts a totally α-helical structure, whereas the structure of melittin is mainly α-helical and presents some unordered domains. The $L_{15}K_7$ α-helix axis is oriented essentially parallel to the air-water interface plane; it differs for melittin. When injected into the subphase, $L_{15}K_7$ and melittin insert into preformed expanded DMPC monolayers and can be detected by PMIRRAS, even at low peptide content (>50 DMPC molecules per peptide). In such conditions, peptides have the same secondary structure and orientation as in pure peptidic films.

INTRODUCTION

Interactions of peptides with lipids in biological and model membranes have been studied by different spectroscopic techniques, such as circular dichroism, NMR, and Fourier transform infrared (FTIR) spectroscopy (Harris and Chapman, 1992; Surewicz et al., 1993). In principle, infrared spectroscopy is well suited to give simultaneous information on both membrane components, but up to now, such studies have been carried out only in solution (Dousseau and Pézolet, 1990; Arrondo et al., 1993) or on thick (Goormarghtigh et al., 1990) and Langmuir Blodgett films (Briggs et al., 1986) by attenuated total reflection (ATR) spectroscopy. Data obtained from solution cannot account for the relative orientation of the bound peptide versus the phospholipid molecules, whereas ATR measurements provide such information, but only for rehydrated films with minimal water content. As recently documented (Frey and Tamm, 1991), low water content induces modifications in peptide position, as compared to fully hydrated systems. Measurements of a fully hydrated state, such as Langmuir films, thus appear to be more appropriate.

Monolayers at the air-water interface provide a unique model for amphipathic molecules. They assemble spontaneously and their packing constraints can be varied conveniently by changes in the lateral pressure and the physicochemical conditions in the subphase. Such possibilities have already been extensively used for lipid and protein films. For amphipathic peptides this approach is especially well adapted because the first step in their mode of action is always an interaction with the membrane interface. Moreover, it is clear today that we need to know how such amphipathic peptides can pack together in membranes. At least two states can be considered: they can stand flat at the interface or make helix bundles and orient perpendicular to it (Cornut et al., 1993; Matsusaki et al., 1995; Wu et al., 1995; Zhang et al., 1995). The structure of peptides in pure peptidic films offers new conditions were such peptide packing can be studied. This will afford a look at different constraints, as compared to the only situation where peptide packing is known, the crystal state (which, unfortunately, occurs in very few cases). Finally, from a spectroscopic point of view it is a requirement to study each of the components before being able to address the problem of their structure in a mixture.

Owing to their amphipathic properties, peptides such as melittin (Mel) (Colaccico et al., 1977; Bhakoo et al., 1982), δ-hemolysin (Bhakoo et al., 1982), bombolitins (Signor et al., 1994), signal peptides (Briggs et al., 1986), and proteins like apoliproteins (Krebs and Phillips, 1984), cardiotoxins (Bougis et al., 1981), and membrane proteins (Jones and Davies, 1987) form rather stable films at the air/water interface. To our knowledge, no structural study has been performed until now on such peptidic films in situ. The strong absorption of the water vapor and the poor detectivity of conventional FTIR spectroscopy led to the discarding of this technique for the study of such interfacial systems. Despite these difficulties, Dluhy et al. (1988, 1989) managed to detect in situ pure phospholipid monolayers on a Langmuir trough by infrared reflection (IRRAS). Recently, differential reflectivity measurements by polarization modulation of the incident light (PMIRRAS) has proved to be an

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efficient way to greatly increase the surface absorption detectivity while getting rid of the intense isotropic absorptions occurring in the sample environment (Blaudez et al., 1993).

In this work, we used PMIRRAS to show that the conformation and the orientation problems can be addressed in situ for pure lipidic and peptidic films as well as for mixed peptide-lipid systems. For this purpose, we used Mel, a natural hemolytic peptide, and $L_{15}K_7$, a synthetic 22-residue-long amphipathic peptide constituted only by apolar Leu and charged Lys residues. These peptides were proved to fold mainly into an α-helix (Cornut et al., 1993, 1994). $L_{15}K_7$, 5–10 times more hemolytic than Mel (Cornut et al., 1994), constitutes a model for cytotoxic amphipathic peptides invading natural membranes (Cornut et al., 1993).

MATERIALS AND METHODS

Materials

$L_{15}K_7$ belongs to a series of highly amphipathic synthetic peptides folded into an α-helix in aqueous solution. The $L_{15}K_7$ was synthesized by the solid-phase method using an Fmoc strategy on an automated peptide synthesizer (Cornut et al., 1994). It was purified by HPLC on a CN column, and its composition and sequence were checked by amino acid analysis. It has the following sequence: KLLKLLLKLLKLLLKLLLKLLK. Mel was purchased from Serva (Heidelberg, Germany) and dimyristoylphosphatidylcholine (DMPC) from Avanti Polar Lipids (Birmingham, AL).

Phospholipids were solubilized in chloroform at the concentration of 2 mg/ml. Mel and $L_{15}K_7$ were solubilized in methanol or in water in the concentration range of 0.5 to 3 mM.

Film formation

Langmuir troughs of different sizes were used, depending on the experiments to be performed. They were filled with ultrapure water (Milli-Q, Millipore) with a measured resistivity of 18 $M\Omega\ cm^{-1}$ and a pH of 5.8. Temperature was always maintained at 20°C. Lateral pressure of the Langmuir films was controlled with a Wilhelmy Pt plate. Phospholipid monolayers were studied at different lateral pressures by moving a mobile barrier, in a trough with a 120 cm^2 surface and a 83 cm^3 volume. Peptidic and mixed films were studied in a smaller trough (47 cm^2) with a fixed barrier to reduce the subphase volume to 15 cm^3.

Pure phospholipid films were formed by deposition with a micropipette at the cleaned air-water interface of a few microliters of organic solution, and pure peptidic films wer formed by injection of small volumes of an aqueous solution into the subphase.

To obtain mixed peptide-lipid films, pure DMPC was first spread at the interface at a defined pressure. In a second step a few tens of microliters of concentrated peptide solution were injected into the subphase and the pressure was allowed to equilibrate for about 20 min.

FTIR spectroscopy measurements

Absorbance spectra of pure lipids or peptides as bulk samples were obtained either from powder under the IR microscope, or by conventional transmission study from their organic solution evaporated on a ZnSe window. Spectra of mixed systems as bulk solid samples with different concentration ratios were obtained by conventional transmission study on a BaF_2 plate on which droplets from a mixture of different volumes of lipid and peptide organic solutions have been deposited and evaporated.

Fourier transform infrared spectra were recorded on a Nicolet 740 spectrometer equipped with a HgCdTe detector cooled at 77 K, by co-addition of 200 scans at a resolution of 4 cm^{-1}. A detailed description of the PMIRRAS set-up and experimental procedure has been already given (Blaudez et al., 1994). The infrared beam was polarized by a ZnSe grid polarizer and modulated by a ZnSe photoelastic modulator (PEM) between polarization (p) in the plane of incidence and polarization (s) perpendicular to this plane. For all of the experiments the PEM was set to give halfwave retardation at 7 μm wavelength. A device made of two gold mirrors and one ZnSe lens allowed back-and-forth reflections of the beam at the air/water interface and its focus on the detector (Fig. 1). Optimal conditions for detection have been obtained with an incidence angle of about 75° relative to the normal to the water surface.

The PMIRRAS signal can be expressed (Blaudez et al., 1994) as follows:

$$S = C\frac{(R_p - R_s)}{(R_p + R_s) + J_0(\phi_0)(R_p - R_s)}J_2(\phi_0)$$

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 maximun dephasing given by the photoelastic modulator, and C is a constant that depends on the electronic device.

Optically, in the mid-infrared range, water behaves as a dielectric substrate and therefore contributes to the PMIRRAS signal. Then to extract the weak absorptions bands of the film and to get rid of the dependence on Bessel functions: the monolayer spectrum is divided by that of pure water. In these normalized spectra, the direction of the bands versus the baseline is an indication of the orientation of the transition moment at the water surface and hence of that of the molecular groups themselves. For an incidence angle of 75° it has been determined (Blaudez et al., 1994) that an upward-oriented band indicates a transition moment preferentially in the plane of the surface, whereas a downward-oriented band reveals an orientation preferentially perpendicular to the surface. For a given oscillator strength, bands associated with transition moments parallel to the surface are more intense compared to those perpendicular. When the angle θ between the transition absorption moment and the interface is varied the absorption vanishes at $\theta = 40°$. This value is determined by optical parameters, essentially by the refractive index of the subphase.

RESULTS

Phosphatidylcholine monolayers

The spectrum of bulk DMPC (Fig. 2 *a*) exhibits the characteristic vibrations of phospholipids (Table 1). In agreement with the assignment already reported in the literature (Casal and Mantsch, 1984), the four bands at 1255, 1093, 1063, and 969 cm^{-1} are associated, respectively, with the

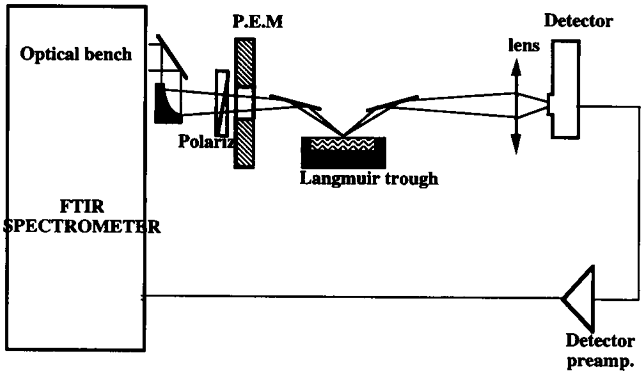

FIGURE 1 Schematic diagram of the PMIRRAS set-up as adapted from Blaudez et al. (1993).

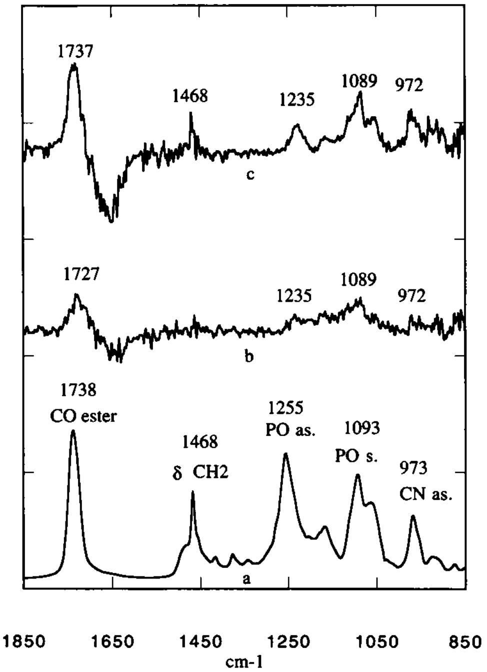

FIGURE 2 IR spectra of DMPC in different states. (*a*) Absorption spectrum of solid anhydrous lipid on ZnSe window. (*b*) In situ PMIRRAS spectrum of an expanded DMPC monolayer ($\pi \approx 0$ mN/m). (*c*) Same as in *b* for a compressed monolayer ($\pi = 30$ mN/m).

antisymmetric P=O, symmetric P=O, C-O-P, and antisymmetric C-N stretching vibrations. The bands at 1738 and 1468 cm^{-1} can be assigned to the stretching of the C=O ester bonds and to the CH_2 bending mode.

When 15 ml of DMPC solution is deposited on the 120 cm^2 water surface, the 1.4×10^{-8} mol of phospholipids forms a very expanded Langmuir film, $\pi \simeq 0$ mN/m, leading to a 140 Å^2 area per molecule. The spectrum of this monolayer is presented in Fig. 2 *b*. The broad dip at 1650 cm^{-1} is a general feature of such spectra; it corresponds to different optical responses of the covered and uncovered water surface and to spectral contribution of the water subphase (Blaudez et al., 1993, 1994). The main bands of the film (Table 1) appear upward relative to the baseline, which indicates that their transition moments are preferentially in the surface plane.

The stretching $\nu_{C=O}$ band is redshifted by 11 cm^{-1} compared to its position in the bulk (Table 1). This indicates that, at this very low lateral pressure, ester groups interact with the aqueous phase through hydrogen bonds. The high intensity of this band suggests a favorable orientation of the C=O bond in the plane of the film. Moreover, its halfband width (56 cm^{-1}) is larger than that observed with anhydrous DMPC (32 cm^{-1}), which suggests a disorder of the polar groups at the water surface.

Similarly, redshifts between the bulk and the film spectra are observed for the bands associated with the antisymmetric (30 cm^{-1}) and symmetric (3 cm^{-1}) $\nu_{P=O}$ stretching vibrations (Table 1). According to the literature (Ter-Minassian-Saraga et al., 1988; Wong et al., 1988), this reveals the formation of hydrogen bonds between water and the phosphate groups.

The very low intensity of the δ_{CH2} band at 1470 cm^{-1}, compared to its value in the bulk state, indicates a large disorder in the hydrocarbon chains. The δ_{CH2}, δ_{CH3} sym, and $\nu_{C\text{-}O}$ ester absorption bands are not observed on the Langmuir film spectra, probably because of their inherent weak intensity.

When compressing the DMPC film to a lateral pressure of $\pi \approx 30$ mN/m, bands increase in intensity and sharpen (Fig. 2 *c*). The same behavior is observed in the ν_{CH2} stretching region (Fig. 3), in agreement with what has been already reported (Dluhy et al., 1989). This effect, well documented for long chain fatty acids, is indicative of a quasi-crystalline organization of the hydrocarbon chains in the monolayer.

At this high lateral pressure, the $\nu_{C=O}$ band recovers the same frequency position as for the bulk state (Table 1). This suggests a decrease in the interactions between C=O ester groups and the water subphase when lateral pressure increases. However, the width of this band, not as sharp as in the bulk, indicates a residual disorder even at such high pressures.

At 973 cm^{-1}, the antisymmetric $\nu_{(CH3)3\text{-}N}$ stretching band is oriented upward and is rather intense. The transition moment of this vibration is perpendicular to the C_3 axis of the the trimethylammonium group, and this suggests that this C_3 axis takes a peculiar orientation perpendicular to the interface.

Peptidic films

The spectra of $L_{15}K_7$ and Mel bulk samples are shown in Fig. 4, *a* and *c*. Three main amide bands I, II, and III are detected, respectively, around 1650, 1540, and 1293 cm^{-1}. These values are in good agreement with those already found in the literature for Mel (Lavialle et al., 1982; Weaver et al., 1992) and related peptides (Jackson et al., 1992; Zhang et al., 1992; Brauner et al., 1987). The intensity ratio between the amide I and the amide II bands is about 1.5. All such features are consistent with an α-helical structure.

Spectra of peptidic films can be detected in situ at very low concentrations in the subphase, i.e., at about 1 μM for $L_{15}K_7$ and 0.5 μM for Mel. The best spectra shown in Fig. 4, *b* and *d*, were obtained for a deposit of 30–40 ml of the organic solution of peptide (about 2–3 10^{-8} mol), which resulted in a lateral pressure in the range of $\pi \sim 5$–10 mN/m. The spectrum of $L_{15}K_7$ at the interface (Fig. 4 *b*) displays essentially one strong and sharp absorption band at 1657 cm^{-1}, which can be unambiguously associated with the amide I mode (Table 1). According to the literature (Dousseau and Pézolet, 1990; Arrondo et al., 1993), the sharpness and position of this band allow us to conclude that the peptide adopts a pure α-helical structure at the interface. It should be emphasized that the amide II band has almost

TABLE 1 Frequencies and attributions of the main vibration bands for lipids, peptides, and lipid-peptide monolayers

cm^{-1}	ν C=O ester	Amide I	Amide II	δ-CH_2	ν P=O antisym.	ν P=O sym.	ν C-O ester	ν C-N antisym.
DMPC bulk	1738			1468	1255	1093	1063	973
DMPC expanded film	1727			—	1235	1089	—	972
DMPC compressed film	1737			1468	1235	1089	1963	972
Peptides bulk Mel, $L_{15}K_7$		1653	1540					
Mel film		1656	1542					
$L_{15}K_7$ film		1657	1536					
Mel/DMPC mixed film	1743	1670	1540	1468	1253	—	—	—
$L_{15}K_7$/DMPC mixed film	1732	1658		1465	1253	1085	—	980

totally disappeared, indicating that the intensity ratio of the amide I to amide II bands (A_I/A_{II}) is higher than that in the bulk state (Fig. 4 *a*).

The spectrum of Mel at the interface (Fig. 4 *d*) differs significantly because it exhibits both amide I and II bands at 1656 and 1542 cm^{-1}, with an intensity ratio $A_I/A_{II} = 2.6$. These data are in agreement with a general folding of Mel in the α-helix. However, the simultaneous presence of the amide I and II bands suggests that its average orientation relative to the interface differs from that of $L_{15}K_7$. The strong absorption bands detected in the bulk spectrum between 1150 and 1250 cm^{-1} are missing.

Mixed phospholipid-peptide films

Different procedures were carried out to obtain mixed films. Good stability and detectivity were obtained when peptides were added via the subphase to an expanded preformed phospholipid monolayer and not when proceeding on the reverse way, i.e., when adding lipids to a performed peptidic film. When peptide is injected into the subphase, the lateral pressure increases concomitantly. It has been necessary to add about 1.2 to 3.2 × 10^{-8} mol of peptide to a DMPC phospholipid film of 0.6 to 0.9 × 10^{-8} mol to modify significantly the lateral pressure and the spectrum of the pure DMPC monolayer.

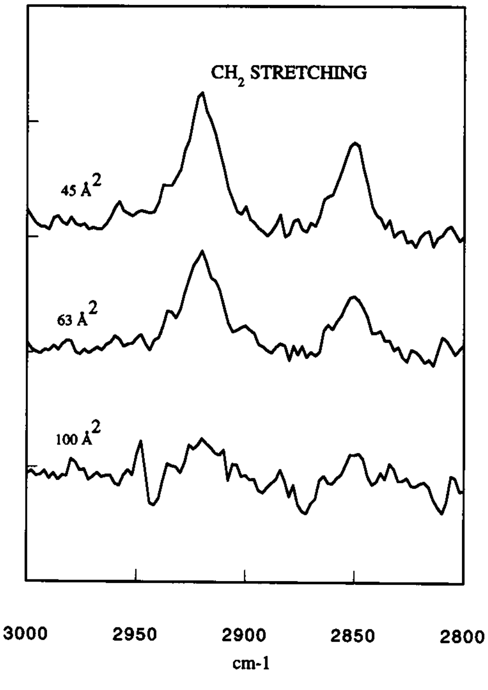

FIGURE 3 In situ PMIRRAS spectra of DMPC at different molecular areas detected in the CH stretching domain.

With $L_{15}K_7$, a stable PMIRRAS spectrum of the mixed film is obtained about 20 min after peptide injection. Successive additions of $L_{15}K_7$ in the subphase allow us to detect spectra with varying lipid-to-peptide composition. The characteristic absorption bands of the lipidic and peptidic components can be identified on these spectra (Fig. 5 *b*). Injection of the peptide into the subphase induces weak changes in the DMPC spectrum. Moreover, on the mixed-film spectra (Fig. 5 *b*) one detects always the amide I band centered at 1658 cm^{-1}, which is better seen on the difference spectrum (b-a) as shown on Fig. 5 *c*. Again the shape and position of this amide I band (Table 1) indicate an α-helical structure, and the absence of the amide II suggests that the helix is oriented as in pure peptidic films.

Changes induced when Mel is injected into the subphase, at a concentration of 0.29 μM, and below a DMPC film compressed at 15 mN/m are shown on Fig. 6. On comparison to the pure DMPC film (Fig. 6 *a*), new absorption bands are detected. The difference between spectra with (Fig. 6 *b*) and without (Fig. 6 *a*) Mel is shown in Fig. 6 *c*. Essentially two bands at about 1670 and 1540 cm^{-1} are detected; they correspond to the amide I and II bands coming from the peptide (Table 1). Therefore, one can conclude that Mel is also present at the interface. The detection of both the amide I and amide II bands at positions rather similar to those on pure peptidic films (Fig. 4 *d*) leads us to propose a similar secondary structure and orientation at the interface.

In all such experiments only the total amount of added peptide is known because it partitions between the film and the subphase. To define the concentration of the peptide in the film, a series of absorbance infrared spectra of lipid-peptide mixtures at various phospholipid-to-peptide molar

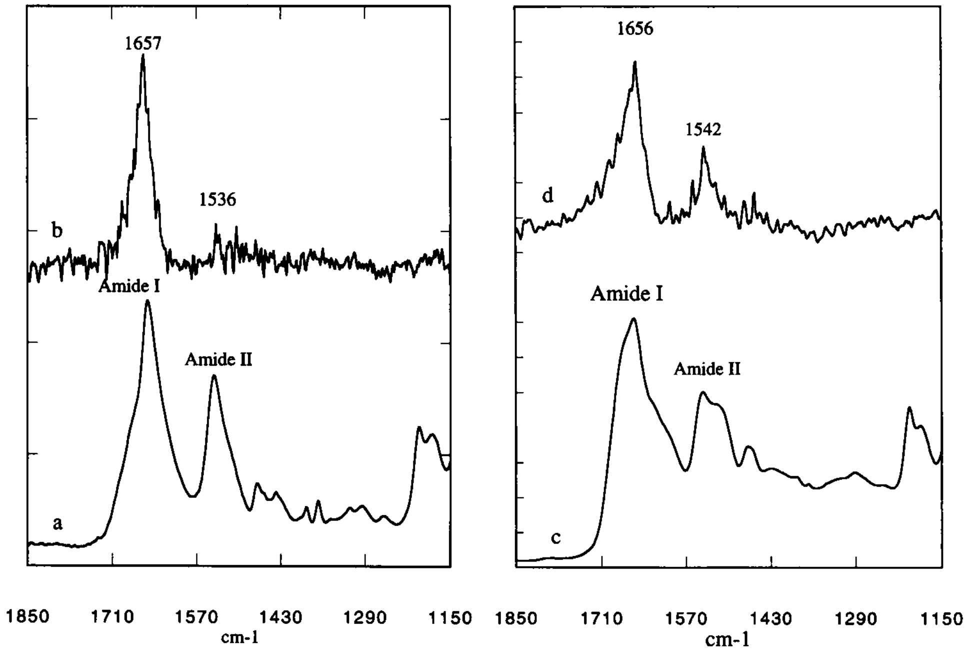

FIGURE 4 IR spectra of peptides in different states. (*a*) Absorption spectrum of $L_{15}K_7$ peptide in solid state. (*b*) In situ PMIRRAS spectrum of $L_{15}K_7$ at the air/water interface (π = 1.7 mN/m). (*c*) Absorption spectrum of Mel in solid state. (*d*) In situ PMIRRAS spectrum of Mel at the interface (π = 3 mN/m).

ratios (R_i) was acquired first. Then, for both peptides, the ratio $R = A_{ester}/A_{amide}$ of the lipid ester and peptide Amide I band intensities was used to construct a diagram versus R_i. As seen on Fig. 7, R increases linearly versus R_i, in agreement with the literature (Goormaghtigh et al., 1994).

In the case of $L_{15}K_7$ mixed films (Fig. 5 *a*), we get the A_{ester}/A_{amide} ratio, which corresponds on the abscissa (Fig. 7) to a lipid-to-peptide molar ratio of 50 in the film. Such a determination could be misleading because of the consequence of preferred orientation of molecules and different absorption coefficients. However, in our case this drawback is limited because of the fact that the absorption transition

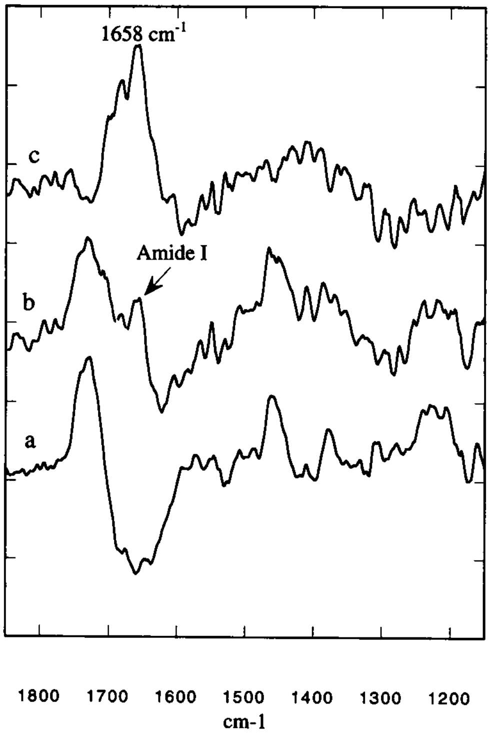

FIGURE 5 PMIRRAS spectra of mixed lipid-$L_{15}K_7$ films. (*a*) Pure DMPC film (π = 19.5 mN/m). (*b*) Mixed lipid-$L_{15}K_7$ film (π = 28 mN/m) after injection in the subphase of $[L_{15}K_7]$ = .70 μM. (*c*) Difference spectrum between spectra *b* and *a*.

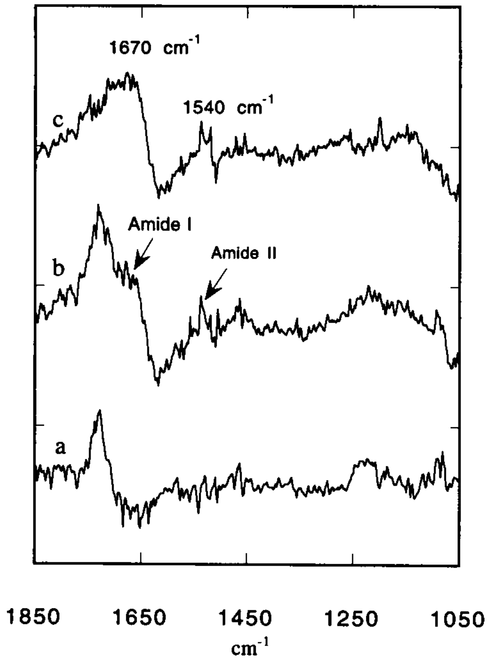

FIGURE 6 PMIRRAS spectra of mixed DMPC-Mel films. (*a*) Pure DMPC film (π = 19 mN/m). (*b*) DMPC-Mel film after injection in the subphase of [Mel] = 1 μM; final pressure, 25 mN/m. (*c*) Difference spectrum between spectra *b* and *a*.

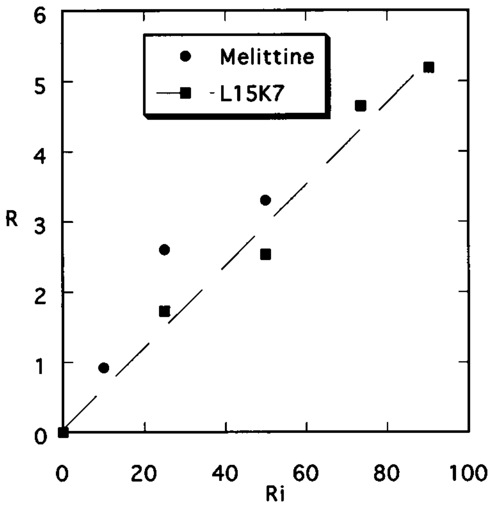

FIGURE 7 Diagram to determine the lipid-to-peptide molar ratio within the film at interface. The absorption ratio, $R = A_{ester}/A_{amide\ I}$ in mixed bulk compounds, is plotted versus the lipid-to-peptide molar ratio R_i for Mel (●) and $L_{15}K_7$ (■).

moments of the amide I and ester vibrations have roughly the same orientation in the film (see Discussion). As a consequence and even at low concentration in the subphase (1 μM), the $L_{15}K_7$ peptide has enough interface affinity to insert into the film in a ratio of one peptide for 50 phospholipid molecules. This also shows that the technique is sensitive enough to detect few molecules when properly oriented at the interface.

Finally, peptide injection into the subphase leads to a lateral pressure increase: for $L_{15}K_7$ it goes from 19.5 to 28 mN/m in the 0.5–2.4 μM concentration range and from 13.5 to 22 mN/m for Mel in the 1–4.2 μM concentration range. This compares well with the increase in lateral pressure, typically $\Delta\pi = 10$ mN/m, already observed in lipid monolayers induced by other amphipathic peptides (Bhakoo et al., 1982; Signor et al., 1994; Bougis et al., 1981; Fidelio et al., 1981) and is another argument to assess peptide insertion.

DISCUSSION

The polarization modulation technique used herein proved to be very sensitive and allowed us to detect the infrared spectra of expanded monolayers of phospholipids, polypeptides, and their binary mixtures, in situ, at the air-water interface.

The DMPC PMIRRAS spectra have a better quality compared to the only ones previously obtained by IRRAS, i.e., without modulation (Dluhy et al., 1988). When pressure increases, successive observations allow us to detect a progressive ordering of the aliphatic chains, in agreement with previous studies (Dluhy et al., 1989). Moreover, information on the orientation of polar groups are obtained.

Peptidic films were detected at the interface, even at low lateral pressure, $\pi \leq 3$ mN/m. Even in such extreme conditions the two peptides studied have rather narrow amide bands at the characteristic position of α-helical structures. Then highly diluted peptides are stabilized at the interface in structures very similar to those documented by CD and FTIR in bulk in more concentrated solutions, i.e., when the peptides are self-associated or in their dried state (Cornut et al., 1994; Lavialle et al., 1982; Weaver et al., 1992). This is particularly clear for $L_{15}K_7$; it was designed to be able to form an ideal amphipathic helix, and the data herein prove it behaves as a pure α-helix lying at the interface.

To understand spectra in terms of α-helix orientation relative to the water surface, the theoretical normalized PMIRRAS signal was calculated for an anisotropic polypeptide monolayer, using a general software program (Buffeteau and Desbat, 1989). We have generated values of the optical anisotropic indices of the film by taking into account the relative infrared absorptions and dichroism reported for the amide I and amide II absorptions in the case of a polypeptide with a α-helix secondary stucture (Tsuboi et al., 1963; Ishida and Griffiths, 1993). The simulation was performed at different orientation angles of the α-helix relative to the water surface; the results are reported Fig. 8. In agreement with the PMIRRAS selection rule at a dielectric interface (Blaudez et al., 1993), when the helix axis is parallel to the interface these simulations give a strong positive amide I band and a weak positive amide II band. Conversely, when the α-helix is perpendicular to the interface, a negative strong amide I band and a strong positive amide II band are expected. It is clear that the experimental PMIRRAS spectrum of $L_{15}K_7$ (Fig. 4 *b*) favors an orientation of the α-helix parallel to the air/water interface.

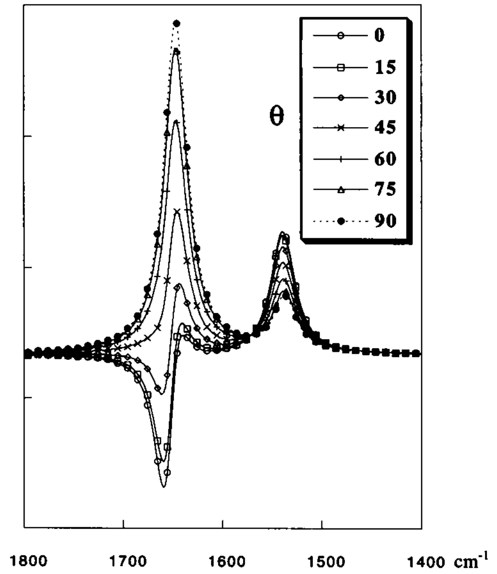

FIGURE 8 Calculated spectra in the amide I and II region for pure α-helices when changing the tilt angle, θ, between the helical axis and the normal to the interface, as indicated in the plot. θ = 90° corresponds to a helix parallel to the interface, θ = 0° to a helix perpendicular to the interface.

In the case of Mel, the film spectra (Fig. 4 *d*) are rather similar to those in bulk and those already reported in the literature (Lavialle et al., 1982). The broadening of the amide I band indicates that secondary structures other than the α-helix are present. This agrees with the general finding of a maximum of 60–76% H_α for tetramers in solution as determined by CD (Talbot et al., 1979; Vogel, 1987). Furthermore, on the PMIRRAS spectra of Mel both amide I and II bands are present. This indicates that the general orientation of the peptide differs from that of $L_{15}K_7$.

When peptides are associated with a DMPC monolayer the peptide spectral features are at first glance very similar to those of purely peptidic films. Then the secondary structure of the peptide is similar to that discussed above and agrees with that determined by CD and FTIR for Mel bound to bilayers (Dempsey, 1990; Weaver et al., 1992) and for $L_{15}K_7$ by CD (Cornut et al., 1994).

Until now the orientation of Mel at the interface of a monolayer was only approached from the molecular area occupied at interface near the collapse pressure. From such studies, an area of about 90 $Å^2$ per molecule was first proposed when the subphase is pure water (Gevod and Birdi, 1984), whereas other authors proposed a value of about 370 $Å^2$ per molecule, which corresponds to a peptide almost fully extended at the interface (De Grado et al., 1981). Recently a similar study of bombolitin, a very similar 17-residue-long cytotoxin, led to the value of 250 $Å^2$ per molecule, which again corresponds to a peptide that is flat at the interface (Signor et al., 1994).

It can be stressed that in the same conditions Mel and $L_{15}K_7$ seem to behave differently, according to orientation. This difference could reflect the different amphipathic topology of both peptides. $L_{15}K_7$ is an ideal amphipathic rod whose stable, flat orientation at the interface could have been anticipated from its sequence and thermodynamical criteria. In contrast, Mel has a strongly polar Cterm hexapeptide segment that should remain hydrated, and an N-term segment that is rather weakly polar, except for the single Lys_7, which is required for activity (Blondelle et al., 1991). The two α-helical segments connected through the Pro_{14} could be more or less tilted compared to the interface. Analysis of Mel orientation requires, therefore, a quantitative interpretation of the absorbancies and definition of the orientations of at least three different components: two helical and the Cterm segments. As mentioned earlier (Vogel, 1987), the measure of a single-order parameter does not allow us to totally discriminate between different models.

The conclusions concerning the orientation of the peptides at the lipid interface are similar to those discussed above for pure peptidic films; they have to be compared to the abundant literature on the subject essentially documented by FTIR ATR (Weaver et al., 1992; Jackson et al., 1992; Zhang et al., 1992). However, as recently reported (Frey and Tam, 1991), Mel orientation appears to be sensitive to the sample preparation and especially to the proper hydration. Indeed, the monolayer geometry and PMIRRAS acquisition of spectra can overcome some drawbacks, but more quantitative standards are needed for amide I and II absorption bands for simple peptides in well-defined single orientations.

The comparison of Mel and $L_{15}K_7$ shows that the latter, which has the higher hemolytic activity (Cornut et al., 1994), is almost exactly parallel to the interface. Very recently the same orientation was proposed from x-ray diffraction for alamethicin diluted on bilayers, one peptide per more than 50 lipid molecules (Wu et al., 1995), despite the fact that this peptide is the more hydrophobic member of such a series of cytotoxic peptides (Cornut et al., 1993). For Mel the same orientation has been proposed in different situations (Dempsey, 1990). For other cytotoxic peptides it has also been shown that they could change their orientation according to the bilayer constraints; this is the case for δ-hemolysin from *Staphylococcus aureus* (E. Thiaudière et al., manuscript in preparation) and for magainin, as recently documented (Ludtke et al., 1994), when the peptide concentration is increased within the bilayers.

In conclusion, these data are the first obtained by vibrational spectroscopy on such systems, in situ, at the air/water interface. Although the present work is a first step, this approach will certainly allow us to gain new information on lipid-peptide interactions. However, it will be necessary to carry out a more systematic study on these systems by changing the nature of the phospholipid, the pH, the salinity of the subphase, and the lateral pressure. But from now on we can get complementary information on the structure of amphipathic peptides alone and inserted in a lipidic matrix. At the moment, orientation of helical segments compared to membranes is an important problem; PMIRRAS on monolayers proved to be an alternative approach that is especially well adapted for water-soluble peptides, or proteins, which should incorporate into lipids. Today simple primary amphipathic peptides of generic sequence such as $Lys_2Leu_nLys_2$ proved to be a good model for transmembrane helical segments (Davies et al., 1983; Zhang et al., 1992, 1995). Leu_iLys_j peptides with a periodic presence of charged residues at each helical turn, like $L_{15}K_7$ studied herein, provide ideal secondary amphipathic helices that can be good models for protein segments that are flat at the membrane surface.

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