Interaction of a Nonspecific Wheat Lipid Transfer Protein with Phospholipid Monolayers Imaged by Fluorescence Microscopy and Studied by Infrared Spectroscopy

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ABSTRACT The interaction of a nonspecific wheat lipid transfer protein (LTP) with phospholipids has been studied using the monolayer technique as a simplified model of biological membranes. The molecular organization of the LTP-phospholipid monolayer has been determined by using polarized attenuated total internal reflectance infrared spectroscopy, and detailed information on the microstructure of the mixed films has been investigated by using epifluorescence microscopy. The results show that the incorporation of wheat LTP within the lipid monolayers is surface-pressure dependent. When LTP is injected into the subphase under a dipalmytoylphosphatidylglycerol monolayer at low surface pressure (<20 mN/m), insertion of the protein within the lipid monolayer leads to an expansion of dipalmytoylphosphatidylglycerol surface area. This incorporation leads to a decrease in the conformational order of the lipid acyl chains and results in an increase in the size of the solid lipid domains, suggesting that LTP penetrates both expanded and solid domains. By contrast, when the protein is injected under the lipid at high surface pressure (≥20 mN/m), the presence of LTP leads neither to an increase of molecular area nor to a change of the lipid order, even though some protein molecules are bound to the surface of the monolayer, which leads to an increase of the exposure of the lipid ester groups to the aqueous environment. On the other hand, the conformation of LTP, as well as the orientation of α-helices, is surface-pressure dependent. At low surface pressure, the α-helices inserted into the monolayers are rather parallel to the monolayer plane. In contrast, at high surface pressure, the α-helices bound to the surface of the monolayers are neither parallel nor perpendicular to the interface but in an oblique orientation.

INTRODUCTION

Among the broad family of lipid transfer proteins (LTPs) found in living organisms, plant LTPs form a unique class of highly homologous proteins. They are characterized by a molecular mass of ~9 kDa, a basic isoelectric point, and a folded polypeptide chain stabilized by four disulfide bridges. They belong to a multigenic family, and the expression of the different isoforms is spatially and temporally regulated (Kader, 1990). Because these proteins facilitate, in vitro, the exchange and transfer of different amphiphilic lipids, it was initially suggested that they were involved in the cell traffic of membrane lipids. This function was rejected after the discovery that these proteins follow the secretory pathway and have an extracellular localization, probably on the cell walls (Tchang et al., 1988; Sossountzov et al., 1991). Then it was proposed that they might participate in the formation of plant cutin by transporting the hydrophobic cutin monomers, which was an attractive hypothesis to reconcile both lipid transfer and extracellular localization of LTPs (Sterk et al., 1991). More recently, it was shown that LTPs could also inhibit, in vitro, the growth of pathogenic fungi and bacteria, thus suggesting that plant LTPs could play a role in the defense mechanism of plants against microbial invaders (Terras et al., 1992; Molina et al., 1993). These different hypotheses clearly underscore the fact that the physiological function of plant LTPs remains to be elucidated but that it is certainly related to their ability to bind or transport amphiphilic lipid molecules. Finally, their relative abundance in plants also makes them good candidates for interesting technological applications of the lipid transfer activity (Record et al., 1993). In addition, it was shown recently that plant LTPs are good foaming agents (Sorensen et al., 1993).

Studies have been conducted to determine the molecular basis of the lipid transfer properties of LTPs, to elucidate their biological function, and to improve their technological uses. These studies were performed on a wheat LTP that is easily isolated from seeds with a relatively good yield (Désormeaux et al., 1992) and on a natural mutant isolated from maize seeds that exhibits higher transfer activities than those of the wheat protein (Petit et al., 1994).

The three-dimensional structure of the wheat LTP has recently been determined by molecular modeling on the basis of solution nuclear magnetic resonance (NMR) spectroscopy (Simorre et al., 1991; Gincel et al., 1994). The polypeptide chain folds in a bundle of four helices packed against a C-terminal peptide having a unique saxophone shape formed by a succession of turns. A hydrophobic cleft, which involves residues located in the C-terminal end of the

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protein, could be a potential site for the binding of a lipid molecule (Gincel et al., 1994). This α-helical structure is essential for the maintenance of the native conformation necessary for lipid binding, and binding of a phospholipid, lysophosphatidylcholine, leads to an increase in the α-helical content of the wheat LTP (Désormeaux et al., 1992). This increase of helicity most likely explains why it is easier to crystallize the wheat LTP with bound lysophosphatidylcholine than without lipids (Pebay-Peyroula et al., 1992). Furthermore, some difference in the helicity of the wheat and maize LTPs should slightly change the conformation of the binding site and might explain the difference in their lipid transfer activity (Petit et al., 1994).

Lipid transfer also involves binding to bilayer interfaces. This has been approached by following the effect of wheat LTP on the thermotropic behavior of dimyristoylphosphatidylcholine and dimyristoylphosphatidylglycerol vesicles (Désormeaux et al., 1992). The transfer protein affects neither the gel state conformational order nor the phase transition temperature of the diacylphospholipids. Only a slight increase of the conformational order of the acyl chain of dimyristoylphosphatidylglycerol in the liquid-crystalline phase was observed. These results suggest that the binding of LTP to the bilayer interface may be attributable mainly to electrostatic interactions between the positive charges of the protein, which are distributed on its surface, and the negative charges of this anionic phospholipid. This behavior can also explain the improvement of lipid transfer activity when anionic lipids are added to zwitterionic liposomes (Petit et al., 1994).

Even though it is clear that LTP interacts with charged lipids by electrostatic interactions, the results obtained so far do not demonstrate clearly that there is a penetration of the protein into the hydrophobic core of the bilayer. In order to assess this issue, we have used lipid monolayers at the air/water interface. This technique is useful for demonstrating the specific interactions between biologically active molecules and phospholipids (Teissier, 1981; Cornell et al., 1989; Fattal and Ben-Shaul, 1993; Johnson et al., 1993). Moreover, it provides direct information on the degree of protein penetration within the lipid layers (Mombers et al., 1980). In the present study, we have investigated the incorporation of the wheat LTP in monolayers composed of the phospholipid dipalmytoylphosphatidylglycerol (DPPG) as a function of the packing density of film. After injection of the protein into the subphase, the insertion of LTP in lipid monolayers has been measured by the increase in surface area at constant surface pressure. This measurement is directly correlated with the amount of protein penetrating the lipid monolayers (Mombers et al., 1980).

In addition, in order to gain more information about the structure of the lipid-protein complexes, we have studied transferred mixed monolayers of LTP-DPPG by polarized attenuated total internal reflectance (ATR) infrared spectroscopy. This technique is an effective tool for investigating the molecular organization of phospholipid-polypeptide complexes in thin films (Fringeli and Günthard, 1981; Brauner et al., 1987; Cornell et al., 1989; Frey and Tamm, 1991). With the use of this spectroscopic approach, we have studied the secondary structure and orientation of wheat LTP in an oriented phospholipid environment as well as the protein-induced alteration of lipid structure and orientational order. Moreover, detailed information on the microstructure of the LTP-DPPG monolayer has been investigated by using epifluorescence microscopy. This technique is a sensitive optical tool for analyzing the physical behavior of monolayers at the air/water interface (Lösche et al., 1983; Peters and Beck, 1983; Weis and McConnell, 1984) as well as for observing protein binding to lipid monolayers (Blankenburg et al., 1989; Grainger et al., 1989; Ahlers et al. 1992). In the present study, the effect of LTP on the growth of negatively charged DPPG domains formed in the phase transition from the liquid-expanded to the solid-condensed state has been investigated. The results establish a relationship between the size of the lipid domains and the profile of the infrared bands resulting from the C-H stretching vibrations that are sensitive to the conformational order of the lipid acyl chains. Moreover, the presented data provides evidence that LTP partly penetrates the lipid monolayers and that this penetration is strongly dependent on the lipid molecular packing.

MATERIALS AND METHODS

Materials

LTP was purified from wheat seeds (*Triticum aestivum*), as described previously (Désormeaux et al., 1992). Labeled LTP was prepared by reaction of LTP and rhodamine X isothiocyanate (XRITC) (Molecular Probes, Eugene, OR) (XRITC label/protein, 2:1) in sodium bicarbonate buffer (50 mM, pH 9), using the method of Nargessi and Smith (1986). Labeled LTP was separated from unreacted label and buffer salts on Sephadex G-25 M equilibrated in pure water and lyophilized. XRITC-LTP was stored at −20°C until use. The sodium salt of DPPG and egg phosphatidylethanolamine with a fluorescein headgroup (egg PE-fluorescein) were purchased from Avanti Polar Lipids (Alabaster, AL), whereas N-(Texas red sulfonyl)-1,2-(cis-9-octadecenoyl)-*sn*-glycerol-3-phosphoethanolamine, triethylammonium salt (Texas red DOPE) was bought from Molecular Probes and used without further purification. Ultrapure water from a Milli-Q apparatus (Millipore, Bedford, MA) was used to prepare all solutions.

Formation and deposition of Langmuir-Blodgett films

Monolayer experiments were performed in a Teflon trough (15 cm width × 24 cm length × 1.5 cm depth; total subphase volume of 540 ml) using a KSV 3000 Langmuir film balance system. DPPG solution (1.0 mg/ml) was prepared in chloroform/methanol (9:1, v/v) and spread on the subphase (10 mM phosphate buffer, pH 7) with use of a microsyringe. After evaporation of the organic solvent, the DPPG film was compressed to the desired surface pressure at a speed of 3 Å^2/molecule·min. An aqueous solution of LTP (400 μl of a 1 mg/ml protein solution in 10 mM phosphate buffer, pH 7) was then injected into the subphase, and the increase in molecular area was monitored at constant surface pressure. After incubation for 2.5 h at constant surface pressure, the film was transferred onto an ATR germanium crystal using the Langmuir-Blodgett technique. The transfer ratio was always equal to 1.00 ± 0.02. In order to check for protein adsorption from the subphase onto the solid substrate, control experiments

were performed as follows. A lipid-protein film was formed as described above, and the ATR crystal was immersed in the aqueous subphase for the entire incubation time, i.e., 2.5 h. After aspiration of the lipid-protein film with a suction pipette, the ATR spectrum of the subphase revealed the presence of LTP, suggesting that bulk protein was adsorbed on the germanium plate. On the other hand, if the germanium crystal was immersed in the aqueous subphase for only 15 min before the transfer, the ATR spectrum obtained after aspiration of the interfacial film did not reveal any protein signal. Therefore, to be sure that the protein transferred on the germanium plate was bound to the monolayer, the ATR crystal was immersed for only 15 min before the transfer for all spectra shown below. Film depositions were made at a speed of 5 mm/min. The germanium crystal was previously washed with a nonionic detergent (Triton × 100, Sigma, St. Louis, MO), thoroughly rinsed with pure water, and finally rinsed with ethanol. Before use, it was placed in a plasma cleaner sterilizer (model PDC-3X G; Harrick Scientific Co., Ossining, NY) for 5 min. All experiments were carried out at 22 ± 1°C and were reproduced at least three times.

Infrared spectroscopy

Infrared spectra were recorded with a Nicolet Magna 550 Fourier transform infrared spectrometer (Nicolet Instrument Corp., Madison, WI) equipped with a liquid nitrogen cooled mercury-cadmium-telluride detector. The instrument was continuously purged with dry air. The crystals on which the dried monolayers were transferred were placed in a variable angle vertical ATR accessory (model TMP-220; Harrick Scientific Co.). Germanium ATR parallelograms (50 × 20 × 2 mm with a 45° face angle) were used as internal reflection elements, yielding 25 internal reflections. For polarization measurements, a motorized polarizer (Graseby-Specac, Fairfield, CT) was placed in front of the ATR unit. For each spectrum, 350 scans were co-added and Fourier-transformed to give a resolution of 4 cm^{-1}. All data manipulations were performed with Spectra Calc software (Galactic Industries Corp., Salem, NH). Carbonyl region spectra of the lipid were Fourier-deconvolved by using a narrowing parameter (γ) of 2.25 and an apodization filter of 0.35 (Griffiths and Pariente, 1986).

The lipid/protein molar ratios of the transferred lipid-protein monolayers have been estimated using a calibration curve obtained from the ATR spectra of thick films of DPPG-LTP mixtures of known molar ratios. The samples were prepared by slowly spreading aqueous samples with a Teflon bar on one side of germanium ATR plates until dry films were obtained (Fringeli and Günthard, 1981).

Dichroic ratios (R^{ATR}) of the infrared bands were obtained from the ratio of the peak height intensity of the bands measured with the incident radiation polarized parallel and perpendicular with respect to the plane of incidence ($A_\parallel/A_\perp$) (Fringeli and Günthard, 1981):

$$R^{ATR} = \frac{A_\parallel}{A_\perp} = \frac{E_x^2 k_x + E_z^2 k_z}{E_y^2 k_y} \quad (1)$$

where E_x, E_y, and E_z are the components of the electric field amplitudes, and k_x, k_y, and k_z are the integrated absorption coefficients in the x, y, and z directions, respectively. Assuming a uniaxial fiber-type distribution of orientation with respect to the normal to the ATR crystal, the order parameter $f(\theta)$, relating the orientation of the fiber axis and the normal to the ATR crystal, were calculated by using the following formula (Frey and Tamm, 1991):

$$f(\theta) = \frac{E_x^2 - R^{ATR}E_y^2 + E_z^2}{X\left(\frac{3\cos^2\alpha - 1}{2}\right)(E_x^2 - R^{ATR}E_y^2 - 2E_z^2)} \quad (2)$$

where α is the angle between the transition moment of a given vibration and the fiber axis, θ is the angle between the fiber axis and the monolayer normal, X is the fraction of residues that contributes to the measured signal, and $f(\theta)$ is the order parameter (Fraser, 1953). For thin films, the components of the electric field amplitudes are equal to 1.41, 1.46, and 0.73 for E_x, E_y, and E_z, respectively, using refractive indices of 1.44, 4, and 1 for the thin film, the germanium ATR plate, and air, respectively, and an angle of incidence of 45°(Fringeli and Günthard, 1981).

In order to determine the lipid order parameter $f(\theta)$, the angle α was set to 90° for the symmetric and antisymmetric CH_2 stretching modes of the lipid acyl chains (Fringeli and Günthard, 1981) and to 0° for the carbonyl stretching vibration of the lipid ester groups (Hübner and Mantsch, 1991). Because all methylene segments and all carbonyl groups contribute to the measured signals, X is equal to 1 in Eq. 2.

The order parameter of the α-helical part of the protein in the monolayer has been deduced from the measured dichroic ratio of the amide-I band. In Eq. 2, X is the fraction of amino acid residues in α-helical conformation, and α is the angle of the orientation of the transition moment of the amide I with respect to the helix axis. A value of $\alpha = 39°$ has been used in this work (Tsuboi, 1962), although lower values (24–28°) have been reported (Rothschild and Clark, 1979).

The mean angle $\langle\theta\rangle$ was then calculated from the second Legendre polynomial (Fraser, 1953):

$$f(\theta) = \left(\frac{3\cos^2\theta - 1}{2}\right) \quad (3)$$

Epifluorescence microscopy

The epifluorescence microscope and the associated film balance used in this study are very similar to those described by Meller (1988, 1989) and have been thoroughly described elsewhere (Maloney et al., 1995). Phospholipid monolayers containing a small amount of either Texas red DOPE (for nonlabeled LTP) or egg PE-fluorescein (for XRITC-labeled LTP) lipid probe (0.5 mol%) were compressed to the desired surface pressure, 40 μg of either labeled or unlabelled LTP was injected into the subphase (trough size: 2.5 cm width × 20 cm length × 0.3 cm depth; total subphase volume of 15 ml), and they were incubated for 2.5 h. The monolayer was observed directly, with the epifluorescence microscope mounted above the Langmuir film balance. When the labeled protein was used, the field was observed alternately through two interchangeable cutoff filters, corresponding to signals from the fluorescein-labeled monolayer and XRITC-labeled LTP. A video camera was attached to the microscope, and the images were recorded on a videotape cassette.

RESULTS

Incorporation of LTP into lipid monolayers

In order to test the ability of LTP to penetrate natural or artificial lipid layers, we first studied its surface activity at the air/water interface. It is known that different parameters such as the subphase pH, the ionic strength, or the spreading solvent are important parameters that influence the stability of air-water interface films and prevent solubilization of the protein in the bulk phase. In our case, we were interested in determining the surface properties of the LTP film by using the same subphase as that used to characterize LTP-DPPG films. Thus, we have investigated the effect of the spreading solvent on the surface behavior of LTP. Our results (not shown) showed that unlike the molecular area, the collapse pressure is independent of whether the film was spread from an aqueous solution or from organic solvents. These results suggest that the physical nature of LTP films is the same whatever the spreading solvent. Similar results have been reported for cytochrome *c* spread at the air-water interface (Lamarche et al., 1988). The authors showed that the pro-

cedure used to spread cytochrome *c* does not affect the physical properties of the resulting film and that the only parameter affected is the amount of protein remaining at the interface. Because in our experiments the surface-pressure isotherms of LTP were more reproducible when the protein was spread from an aqueous ethanol solution (ethanol/H_2O, 1:1), this isotherm was chosen as the reference.

Curve *a* in Fig. 1 shows the pressure-area isotherm of pure LTP spread directly at the interface as an insoluble monolayer. This isotherm begins at 360 Å^2/molecule and reaches 210 Å^2/molecule at a collapse pressure of 20 mN/m. Solubilization of the protein into the subphase most likely occurs above this surface pressure. This value is of the same order of magnitude as that found for melittin (Fidelio et al., 1986) and is 9–10 mN/m higher than that obtained for other water-soluble proteins such as cytochrome *c* (Lamarche et al., 1988). This result suggests that LTP is a good surface-active agent and demonstrates its ability to spontaneously penetrate a lipid-free interface. The penetration of the protein into DPPG monolayers has then been studied above and below this value of collapse pressure. After injection of the protein underneath DPPG monolayers, the incorporation of LTP within the lipid monolayer was monitored by the increase of the surface area of the lipid monolayer at constant surface pressure.

Curve *b* in Fig. 1 shows the pressure-area isotherm of the pure DPPG monolayer at the air-water interface. This isotherm presents the three typical regions corresponding to the liquid-expanded region ($\pi < 10$ mN/m), the liquid-expanded to the solid-condensed phase transition region, and the solid-condensed region. It is similar to those previously obtained by Sacré and Tocanne (1977) at pH 6 on 1 mM phosphate buffer or on 100 mM salt. Moreover, the film condensation reaches an area/molecule of 40 Å^2 at a surface pressure equal to 40 mN/m. Again, this value is in good agreement with that previously reported for DPPG monolayers on a subphase pH above 6 (Sacré and Tocanne, 1977). Furthermore, this result is also similar to that obtained from x-ray diffraction studies for the same lipid dispersed in water in the gel phase (40.5 Å^2 at pH 8) (Watts et al., 1981).

Curves *c–f* in Fig. 1 show the compression isotherms of DPPG-LTP films after injection of the protein underneath DPPG monolayers that were compressed to 10 (*c*), 15 (*d*), 20 (*e*), and 35 (*f*) mN/m. As can be seen, the behavior of LTP at the lipid interface is surface-pressure dependent. When the protein is injected into the subphase at a constant surface pressure less than 20 mN/m (Fig. 1, *c* and *d*), the resulting surface pressure isotherm shows that the molecular area of DPPG in the protein-lipid monolayer is very much expanded compared with that of pure DPPG monolayers at the same surface pressure. This increase in molecular area is higher at low surface pressure, being equal to 67 and 26 Å^2/molecule at 10 (curve *c*) and 15 (curve *d*) mN/m, respectively. This result shows that LTP is incorporated into the DPPG monolayer and that the extent of incorporation decreases when the surface pressure of the initial lipid film increases. However, the incorporation of LTP is reversible; this is reflected by the fact that when the mixed LTP-DPPG monolayers are compressed to higher pressures, the compression isotherms tend to converge with that of pure DPPG (curves *c* and *d* compared with curve *b*), suggesting that LTP is progressively squeezed out of the interface. It is noteworthy that these isotherms are shifted to a lower molecular area when the protein is excluded from the interface. This suggests that LTP may drag some lipid in the subphase. On the other hand, when the protein is injected into the subphase at an initial surface pressure of 20 mN/m or above (curves *e* and *f*), little or no change in the film pressure is observed. In fact, the isotherm of pure DPPG and those of mixed lipid-protein films are indistinguishable, indicating that the protein is not incorporated into the film at or above 20 mN/m. These results show that the incorporation of the LTP within the lipid monolayers is surface-pressure dependent and occurs below 20 mN/m.

The composition of the mixed films has been estimated by infrared spectroscopy. Fig. 2 shows the ATR spectra, in

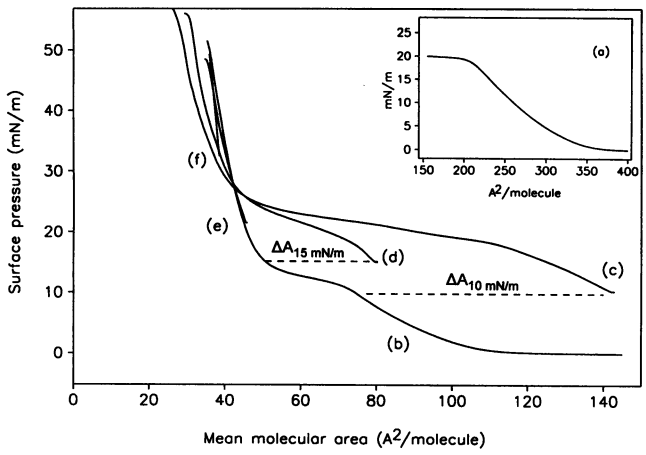

FIGURE 1 Compression isotherms for LTP alone (*a, insert*) and for DPPG monolayers in the absence (*b*) and presence of LTP injected at 10 (*c*), 15 (*d*), 20 (*e*), and 35 (*f*) mN/m.

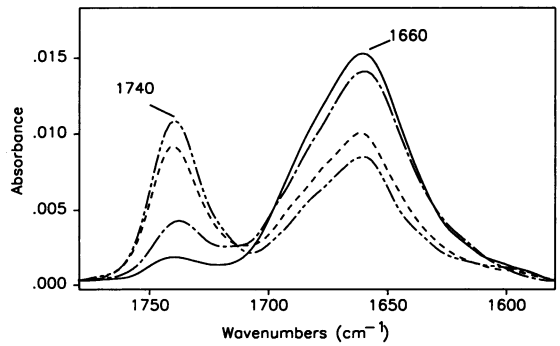

FIGURE 2 ATR infrared spectra, in the 1480 to 1720 cm^{-1} region, of DPPG-LTP monolayers transferred at 10 (——), 15 (— - —), 20 (- - - -), and 35 (— - - —) mN/m.

the 1580–1780 cm^{-1} region, of LTP-DPPG monolayers transferred at 10, 15, 20, and 35 mN/m. This spectral region is characterized by two bands observed around 1740 and 1660 cm^{-1}. These bands are assigned to the C=O stretching vibration of the lipid ester groups and to the amide-I vibration of the protein, respectively (Mendelsohn and Mantsch, 1986). As can be seen in this figure, the intensity of the lipid C=O band increases with the surface pressure, whereas that of the amide-I band decreases. Therefore, the lipid/protein ratio in the mixed LTP-DPPG films increases with surface pressure, in agreement with the observed surface area increase. The lipid/protein molar ratios have been estimated from the intensity of infrared bands resulting from the lipid carbonyl stretching vibration ($I_{C=O}$) at about 1740 cm^{-1} and to the amide-I vibration of the protein ($I_{amide\ I}$) at about 1660 cm^{-1}, using a calibration curve obtained from the ATR spectra of thick films of DPPG-LTP mixtures of known molar ratios between 0 and 50. As can be seen in Fig. 3, the $I_{C=O}/I_{amide\ I}$ intensity ratio varies linearly with the lipid-protein molar ratio of the calibration standards. For mixed monolayers transferred at 10, 15, 20, and 35 mN/m, the lipid/protein ratios interpolated from the curve in Fig. 3 are equal to 3, 8, 30, and 44, respectively. These results demonstrate that LTP can interact with lipid monolayers even at pressures above which it does not penetrate the film, and they reveal the important effect of the packing density of monolayers on the wheat LTP-lipid interactions.

Conformation of LTP bound to lipid monolayers

In order to probe lipid-induced conformational changes of LTP, the spectral region caused by the amide-I vibration (1580–1705 cm^{-1}) has been analyzed. This amide band is known to be sensitive to the conformation adopted by the protein backbone and can thus be very useful in determining protein secondary structure (Dousseau and Pézolet, 1990; Arrondo et al., 1993; Surewicz et al., 1993).

Fig. 4 shows the ATR spectra of LTP-DPPG films transferred at high and low surface pressures (35 and 15 mN/m) as well as the previously reported spectrum of the native protein in aqueous solution (Désormeaux et al., 1992). As can be seen, the binding of the protein to lipid monolayers induces significant spectral changes in the amide-I region. Indeed, the intensity of the bands at 1635 and 1687 cm^{-1}, which are characteristic of amide groups involved in the extended β-sheet structure (Surewicz et al., 1988; Pézolet et al., 1992), relative to that of the 1658 cm^{-1} band caused by the α-helical conformation, is significantly lower in the spectrum of LTP bound to DPPG monolayers compared with that of the native protein. This indicates that the binding of LTP to DPPG monolayers affects the conformation of the protein, as opposed to what has been observed upon binding of LTP to large unilamellar vesicles of diacylphospholipids (Désormeaux et al., 1992).

The surface pressure also seems to affect the conformation of the protein at the interface. The band located at 1658 cm^{-1} in the spectrum transferred at 15 mN/m shifts to 1660 cm^{-1} in the spectrum of monolayers transferred at 35 mN/m, and its width is significantly reduced, suggesting an increase of the α-helical content of the protein at high surface pressure. Although it has not been possible to obtain quantitative results with low prediction errors on the conformation of LTP bound to lipid monolayers because the currently available methods have been developed for proteins in solution (Dousseau and Pézolet, 1990) or for ATR measurements using deuterated protein films (Goormaghtigh et al., 1990), it is clear from the above data that LTP bound to DPPG monolayers is stabilized in the α-helical conformation.

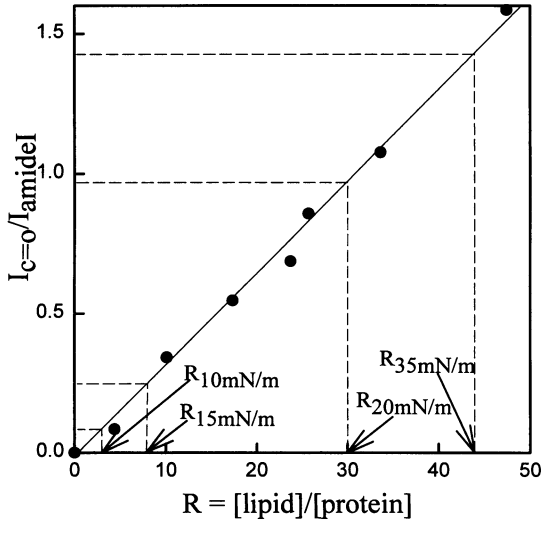

FIGURE 3 Calibration curve for the determination of lipid/protein molar ratios in transferred DPPG-LTP monolayers.

Effect of the incorporation of LTP in lipid monolayers

A microscopic level study

Information on the effect of LTP on the microstructure of the interfacial film was obtained from fluorescence micro-

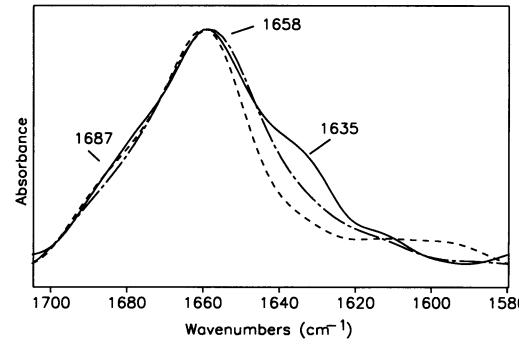

FIGURE 4 Infrared spectra in the amide-I region of LTP in aqueous solution (——) and bound to DPPG monolayers at 15 (— - —) and 35 (- - -) mN/m.

graphs using the fluorescent sulforhodamine lipid probe. For a pressure below 10 mN/m, a homogeneous fluid phase is observed for pure DPPG. Dark domains of rather uniform size, distributed sparsely in the fluorescent fluid phase, appear at higher pressures and have been described as solid lipid domains coexisting with the fluid phase for a variety of phospholipids (Weis and McConnell, 1984; Lösche et al., 1985; Weis, 1991). Fig. 5 presents the micrographs of DPPG films at different surface pressures in the absence (*A–C*) and in the presence (*D–F*) of unlabeled LTP. As can be seen in this figure, the fluorescence images of pure DPPG monolayers show that on increasing the surface pressure from 10 to 20 mN/m (Fig. 5, *A–C*), the size of the lipid domains increases as the proportion of fluid phase diminishes. This behavior seems to be general, and it has been shown that once nucleation has occurred, the solid domains grow in size but not in number as long as the monolayer is steadily compressed (Lösche et al., 1988).

When unlabelled LTP is injected into the subphase of these domains and the surface pressure is maintained constant, the addition of LTP leads to an increase in the size of the domains at surface pressures of 10 and 15 mN/m (compare Fig. 5, *A* and *B*, with Fig. 5, *D* and *E*, respectively). This result suggests that the protein favors the growth of lipid domains. This result is surprising as it is expected that the incorporation of the protein into the monolayer creates disorder in the system, which therefore should prevent lipid crystallization (Rüppel et al, 1982). However, for a surface pressure of 20 mN/m (Fig. 5, *C* and *F*), the size of the domains does not increase, but their shape is strongly affected by the presence of the protein. Indeed, the outer surface of the domains become very fuzzy, with many small protuberances appearing at the surface. It is thus clear that the protein has a strong effect on the lipid monolayer phase behavior when injected in the lipid phase coexistence region. However, one can wonder whether this behavior would be the same when the protein is injected at a surface pressure below the phase coexistence region. Fig. 6, *A–C*, shows that when the protein is injected at a surface pressure below 10 mN/m and further compressed in the phase coexistence region, formation of solid lipid domains is not prevented. The effect of the protein on the lipid domains is similar to that observed at surface pressures of 10 and 15 mN/m (see micrographs in Fig. 5, *D* and *E*, compared with Fig. 6, *A* and *B*, respectively). However, at 20 mN/m, the lipid domains are much larger, and their outer surface is smooth (Fig. 6 *C*) when the protein is injected below the phase coexistence region and is further compressed compared with when it is injected in the phase coexistence region (Fig. 5 *F*). Collectively, these results suggest that the behavior of the phospholipid monolayer is strongly affected by the presence of the protein.

A molecular level study

The effect of LTP and surface pressure on the conformation of the lipid monolayers has been studied using the spectral features associated with the C-H (2800–3000 cm^{-1}), the carbonyl (1680–1780 cm^{-1}), and the phosphate (1150–1300 cm^{-1}) stretching mode regions of the infrared spectra of the lipid. These regions provide valuable structural and conformational information about the changes that occur in the acyl chains, interfacial moiety, and polar headgroup of the lipid molecules, respectively (Casal and Mantsch, 1984; Mendelsohn and Mantsch, 1986).

Fig. 7 shows the acyl chain C-H stretching mode region of the infrared spectra of a monolayer of pure DPPG (- - - -) and DPPG-LTP monolayers (——) transferred at 35 mN/m (Fig. 7 *A*) and 15 mN/m (Fig. 7 *B*). The infrared bands appearing in this region are particularly useful because they are sensitive to the conformation of the phospholipid acyl chains (Unemura et al., 1980). This spectral region is dominated by two strong bands at 2920 and 2850 cm^{-1}, assigned to the methylene antisymmetric and symmetric stretching modes, respectively (Cameron et al., 1980). Weaker bands resulting from the asymmetric and symmetric stretching modes of the terminal methyl groups are also present at 2956 and 2872 cm^{-1}. As can be seen in this figure, the spectra of pure DPPG and DPPG-LTP monolayers at high surface pressure (35 mN/m) are almost identical and are quite similar to the spectrum of DPPG bilayers in the gel state (Babin et al., 1987). These spectra are also similar to those obtained at 20 mN/m (not shown) and to that of the pure DPPG monolayer transferred at 15 mN/m (Fig. 7 *B*, - - - -). This result indicates that at high surface pressure the binding of LTP to DPPG monolayers does not affect the conformation of the DPPG acyl chains. Conversely, at low surface pressure, it is clear that the binding of LTP induces important modifications of the C-H stretching mode region. The two methylene bands become broader and shift toward higher frequency by approximately 1 cm^{-1} in the presence of the protein. Earlier studies showed that these spectral changes are associated with the introduction of *gauche* conformers along the phospholipid acyl chains and the increase of the width of the distribution of conformations (Unemura et al., 1980).

The effect of LTP on the interfacial region of DPPG has also been studied from the carbonyl stretching mode region, which ranges from 1700 to 1760 cm^{-1}. The C=O stretching vibration arising from the ester carbonyl groups of phospholipids is sensitive to hydrogen bonding (Blume et al., 1988). Fig. 8 displays the carbonyl stretching mode region of the deconvolved infrared spectra of DPPG monolayers in the absence and in the presence of LTP at 35 (Fig. 8 *A*) and 15 (Fig. 8 *B*) mN/m. As can be seen, the binding of LTP to DPPG has a marked effect on the spectra of the interfacial region of the lipid regardless of the surface pressure of the film. For pure DPPG, the spectra are characterized by a single band at about 1740 cm^{-1}. Such a band has been observed in the case of dehydrated films of multilayers of diacylphospholipids and has been assigned to nonhydrogen-bonded C=O groups (Hübner et al., 1994). In the presence of LTP, in addition to the 1740 cm^{-1} band, a low frequency component appears at about 1720 cm^{-1} and can be associ-

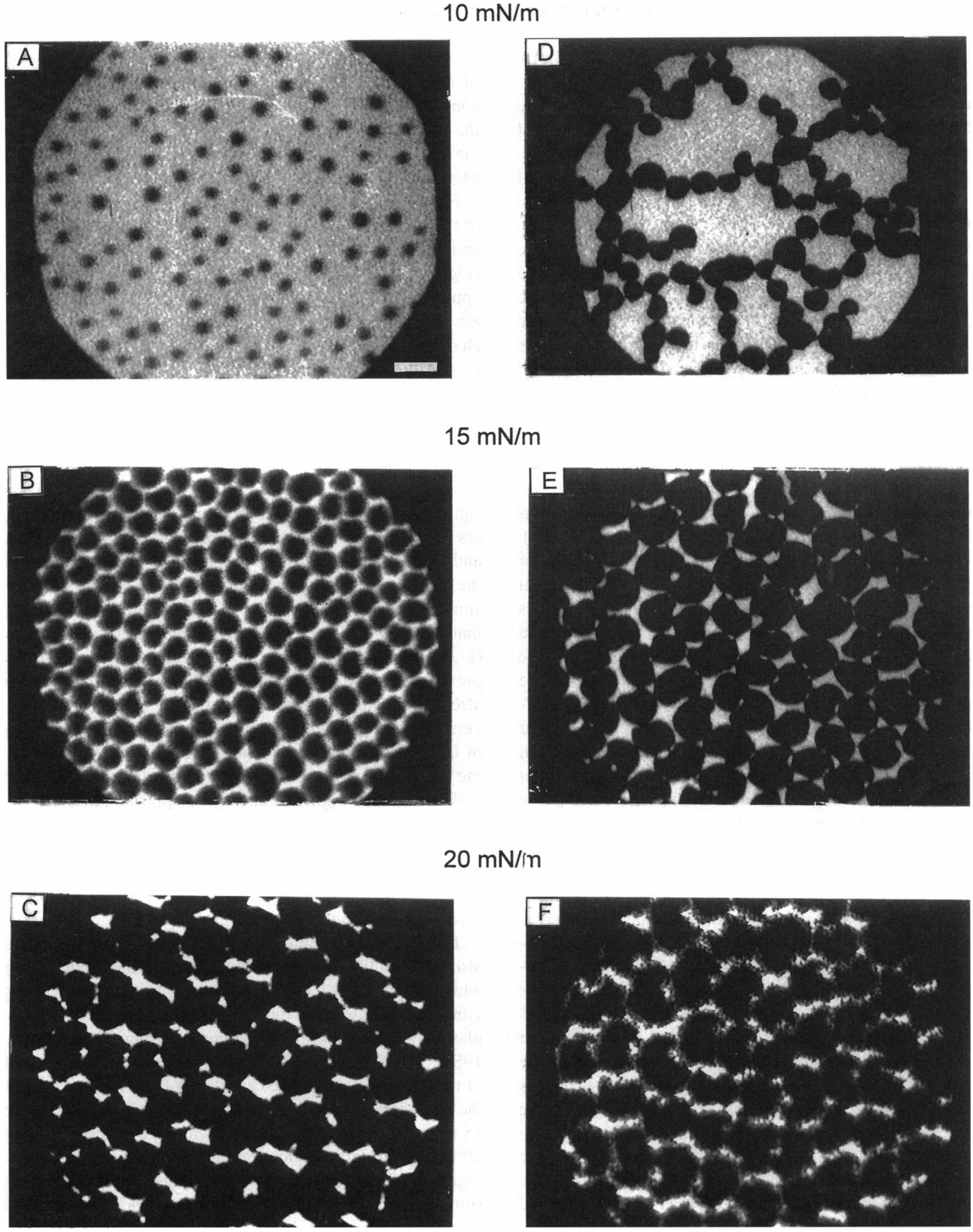

FIGURE 5 Fluorescence micrographs of DPPG monolayers in the absence (*A, B, C*) and presence of LTP (*D, E, F*) at 10 (*A, D*), 15 (*B, E*), and 20 (*C, F*) mN/m. Scale bar in *A* is 20 μm.

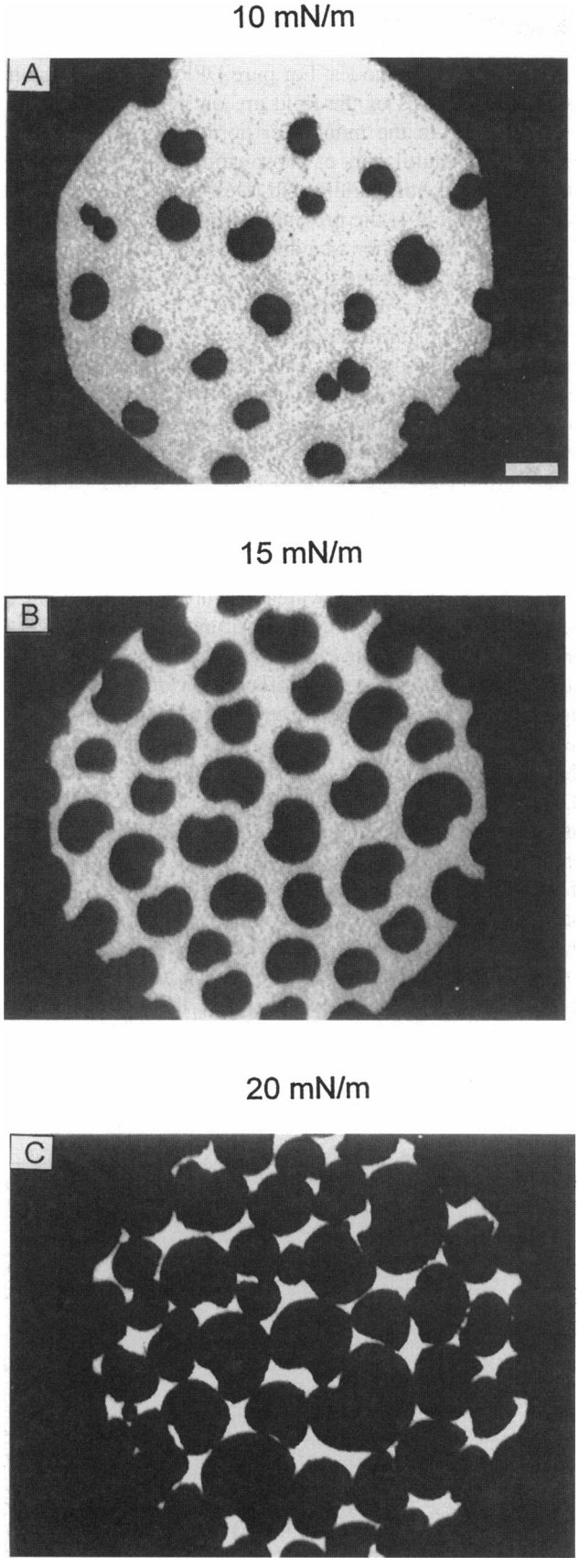

FIGURE 6 Fluorescence micrographs of a mixed LTP-DPPG monolayer compressed at 10 (*A*), 15 (*B*), and 20 (*C*) mN/m. Scale bar in *A* is 20 μm.

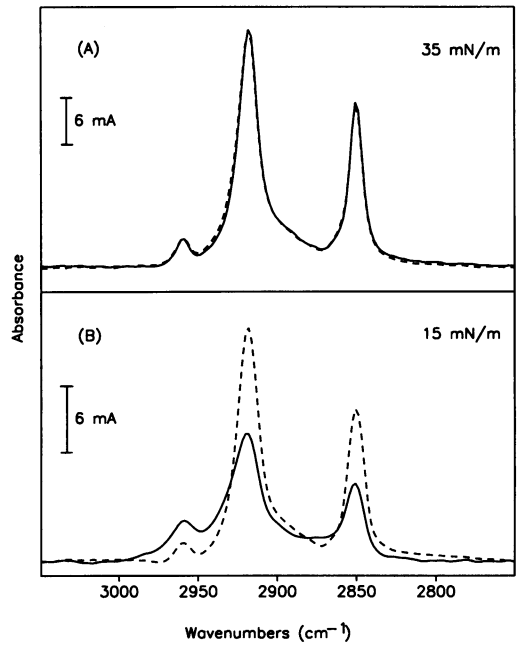

FIGURE 7 ATR infrared spectra in the C-H stretching mode region of pure DPPG monolayers (- - - -) and DPPG-LTP monolayers (——) transferred at 35 (*A*) and 15 (*B*) mN/m.

ated with the presence of hydrogen-bonded carbonyl groups (Blume et al., 1988). As shown in Fig. 8, the effect of LTP on the C=O stretching mode region of the DPPG monolayer is more important for mixed monolayers transferred at low surface pressure than for those transferred at high surface pressure. The relative intensity of the two compo-

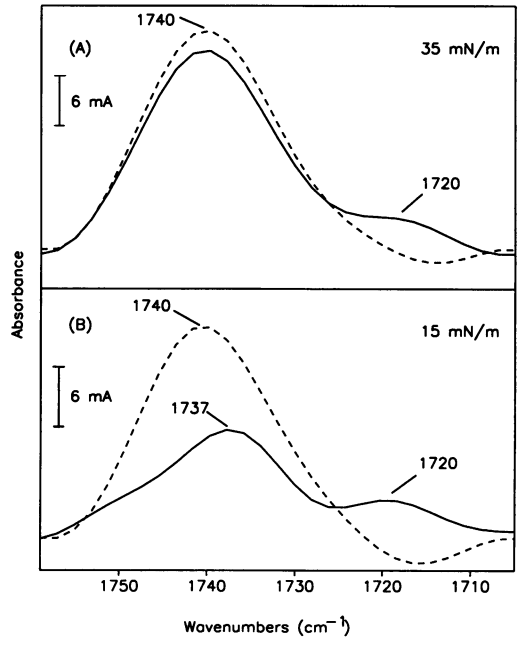

FIGURE 8 ATR Fourier deconvolved infrared spectra of the carbonyl stretching mode region of pure DPPG monolayers (- - - -) and DPPG-LTP monolayers (——) transferred at 35 (*A*) and 15 (*B*) mN/m.

nents of the carbonyl band (I_{1740}/I_{1720}) decreases from 5.2 to 3.2 as the surface pressure decreases from 35 to 15 mN/m, indicating that more carbonyl groups are hydrogen bonded at low surface pressure. In addition to this change, Fig. 8 shows that at low surface pressure, the band caused by free C=O groups is shifted down from 1740 cm^{-1} for the pure lipid to 1737 cm^{-1} for the lipid-protein complex. Such a change could be associated with an increase in the dielectric constant of the environment of some of the C=O groups (Blume et al., 1988) in the neighborhood of the protein molecules.

In order to get more information on the orientation of the protein with respect to the monolayer plane and to assess the effect of LTP on the lipid order, polarized ATR spectra of the transferred monolayers have been recorded. The frequency (ν), dichroic ratio (R), calculated order parameter ($f(\theta)$), and mean angle $\langle\theta\rangle$ of the most relevant infrared bands, measured from three independent experiments, are listed in Table 1 for different surface pressures. As can be seen, the average value of the order parameter for the bands resulting from the symmetric and antisymmetric CH_2 vibrations in the film of pure DPPG monolayers at high surface pressure (35 mN/m) is 0.78. This value suggests that the acyl chains of the lipids are tilted, with an average angle of 22° with respect to the normal to the ATR crystal, assuming that all C-C bonds are in the *trans* conformation. This result is in excellent agreement with the tilt angle of 25° observed for the anhydrous crystal phase of DPPG (Pascher et al., 1987) and indicates that the monolayer is well ordered. Table 1 also shows that the orientation of the methylene chains of the lipids is not affected by the protein at high surface pressure. However, at low surface pressure (15 mN/m), insertion of LTP into the DPPG monolayer produces a substantial decrease in the average order parameter of the CH_2 modes from 0.70 to 0.49. This change is mostly due to the introduction by LTP of *gauche* conformers, as observed from the frequency and width of the bands due to the C-H stretching modes. For pure DPPG monolayers, the ester C=O groups of the lipid are on average oriented at ~62° relative to the monolayer normal, as has also been observed for multilayers of phosphatidylcholine (Hübner et al., 1991) and phosphatidic acid (Désormeaux et al., 1992). As seen in Table 1, the presence of protein seems to affect the carbonyl orientation in a different way that is dependent on the surface pressure. At low surface pressure (≤15 mN/m), the order parameter for the C=O groups varies from −0.17 for pure DPPG film to −0.23 for LTP-DPPG monolayers. This result suggests that the lipid C=O groups are on average oriented at 65° relative to the normal of the crystal plane. Conversely, at a higher surface pressure, LTP leads to a decrease of the tilt angle of the ester C=O groups, which are on average oriented at 61° with respect to the normal to the ATR crystal.

Dichroism measurements of the LTP amide-I region were calculated from the absorbance of the major amide-I band around 1655–1660 cm^{-1}, which was obtained after Fourier deconvolution followed by curve-fitting of the 1600–1700 cm^{-1} region. Although it has not been possible to obtain quantitative results on the conformation of LTP bound to lipid monolayers, infrared spectra show that the fraction of residues in the α-helical conformation is at least equal to that obtained in solution (Désormeaux et al., 1992), i.e., 0.41. Therefore, the order parameter of the helical segments of LTP has been calculated as a function of the surface pressure, using Eq. 2. As seen in Table 1, the orientation of the α-helices is surface-pressure dependent. At low surface pressure (10 and 15 mN/m), the average order parameters of the α-helices of LTP are not so far from the limiting value of −0.5 expected for an α-helix perfectly aligned parallel to the plane of the membrane. In fact, the helix axes are

TABLE 1 Frequency (ν), dichroic ratio (R), average order parameter ($f(\theta)$), and average calculated angle (θ) for selected absorption bands of DPPG and DPPG-LTP monolayers at different surface pressures (π)*

		DPPG				DPPG-LTP			
Assignment	π (mN/m)	ν(cm^{-1})	R‡	$f(\theta)$	$\langle\theta\rangle$	ν(cm^{-1})	R‡	$f(\theta)$	$\langle\theta\rangle$
CH_2 antisymmetric	10	2918.3	0.98–1.01	0.70	26				
	15	2918.4	0.98–1.00	0.70	26	2919.4	1.02–1.06	0.45	
	20	2918.2	0.97–0.99	0.76	24	2917.9	0.95–0.98	0.83	20
	35	2918.1	0.97–0.98	0.77	23	2917.6	0.97–0.98	0.78	22
CH_2 symmetric	10	2850.2	0.97–0.99	0.76	24				
	15	2850.2	0.97–0.99	0.70	26	2850.9	1.00–1.04	0.54	
	20	2850.1	0.97–0.98	0.77	23	2850.1	0.96–0.98	0.82	21
	35	2850.1	0.96–0.97	0.80	21	2850.1	0.96–0.97	0.79	22
C=O	10	1739.3	1.06–1.08	−0.17	62	1737.7	1.02–1.05	−0.23	65
	15	1739.5	1.06–1.08	−0.18	62	1737.7	1.03–1.06	−0.23	65
	20	1739.3	1.03–1.05	−0.23	65	1740.0	1.08–1.11	−0.12	60
	35	1739.4	1.03–1.05	−0.22	65	1739.6	1.06–1.10	−0.16	62
Amide I	10					1659.9	1.07–1.11	−0.36	72
	15					1659.4	1.09–1.1	−0.32	70
	20					1661.2	1.12–1.14	−0.19	63
	35					1660.6	1.13–1.15	−0.15	61

* All values are the average of three independent measurements.
‡ The values indicate the upper and lower limits of R calculated from three measurements.

oriented at about 70° from the normal to the monolayer plane. In contrast, at high surface pressure, the low values of the order parameter of the α-helical segments suggest a random orientation. However, we cannot conclude unequivocally that the change comes from a decrease of the inclination of α-helices or that it can be ascribed to the contribution of randomly oriented molecules that would give the random magic angle of 54.7°.

DISCUSSION

Membrane fluidity plays an important role in lipid transfer activity (Helmkamp, 1983). In the present report, the monolayer technique has been used to study the effect of lipid lateral-packing pressure on the interaction of LTP with lipid membranes. The results presented above show that the protein is a good surface-active agent because its collapse pressure is of the same order of magnitude as that found for the lytic bee venom peptide mellitin (Fidelio et al. 1986), which is considered one of the strongest natural surface-active agents and is described as a "protein detergent." Moreover, once spread at the air/water interface, LTP seems to keep a well structured organization. According to the three-dimensional structure of LTP calculated from molecular modeling (Gincel et al., 1994) on the basis of multidimensional ^{1}H NMR data (Simorre et al., 1991), it was shown that the protein is ovoid in shape, the length of the long and short axis of the ellipsoid of revolution being 28 and 18 Å, respectively. The cross-section for such a structure can thus vary from 395 Å^2, if the protein lies flat on the plane of the interface, to 250 Å^2, if it is oriented perpendicular to the interface. Therefore, the limiting molecular areas of 360 and 210 Å^2 deduced from the protein isotherm suggest that during compression, the orientation of LTP changes from the long axis being parallel to the interface at 0 mN/m to the long axis being perpendicular to the interface at 20 mN/m.

The results obtained on the interaction of LTP with DPPG monolayer clearly emphasize the strong dependence of the packing density of the lipid monolayer on the interaction between LTP and DPPG, and particularly on the penetration of the protein into the monolayer. These results show that there is a limiting surface pressure (~20 mN/m) below which LTP is incorporated into the film, leading to its expansion (Fig. 1). This limiting surface pressure of 20 mN/m is likely related to the collapse pressure of pure LTP (Fig. 1 *a*). The fact that the critical pressure for penetration of LTP into the monolayer is equal to its collapse pressure suggests that in the presence of a DPPG monolayer (Fig. 1, *c* and *d*), LTP exhibits comparable surface activity to that observed for the pure protein. Such a behavior has also been observed previously for cytochrome *c* (Lamarche et al., 1988), which is expelled from the lipid monolayer at its collapse pressure of 12 mN/m.

The relatively greater penetration of LTP into the monolayer at low surface pressures does not necessarily indicate a direct interaction of the protein with DPPG molecules, as LTP alone is surface active and can spontaneously migrate from the subphase to a free-lipid interface. However, the infrared results demonstrate that whatever the surface pressure, interactions occur between the protein and lipid interface. For mixed monolayers transferred at 10, 15, 20, and 35 mN/m, lipid/protein molar ratios of 3, 8, 30, and 44 are deduced from infrared spectra (Fig. 3). Because infrared spectroscopy of the transferred monolayers takes into account both the protein molecules, which penetrate into the monolayer, and those that are bound only to the surface of the monolayer, the above results suggest that at low surface pressure, at least part of the protein molecules penetrate the monolayer, whereas at high surface pressure the LTP molecules are bound only to the surface of the monolayer, inasmuch as no increase in molecular area was detected.

The above results show that different types of interaction between LTP and DPPG monolayers occur, depending on the surface pressure. When the protein is injected under DPPG films at surface pressures of 10 and 15 mN/m, the incorporation of LTP into the films leads to the formation of mixed lipid-protein films. Some of the interactions may not be very strong because a large amount of the protein is excluded from the film upon monolayer compression. Nevertheless, this exclusion of the protein is taking place until the compression isotherm of the LTP-DPPG film (Fig. 1, isotherms *c* and *d*, respectively) converges with that of pure DPPG (Fig. 1, isotherm *b*), which corresponds to a surface pressure of 28 mN/m. This value of 28 mN/m is much higher than the collapse pressure. Therefore, this result strongly suggests that some specific protein-lipid interactions are maintained above 20 mN/m. In addition, this progressive exclusion of the protein does not necessarily suggest that the excluded protein does not interact with monolayer anymore. Moreover, further compression leads to molecular areas lower than that of the pure lipid. At 55 mN/m, areas of 27 and 31 Å^2/molecule are calculated for the protein-lipid film formed at 10 and 15 mN/m, respectively (Fig. 1, isotherms *c* and *d*, respectively). Such molecular areas are impossible if all lipid molecules that initially spread remain at the interface because a typical molecular area of 40 Å^2/molecule should be obtained as it is the case of pure DPPG (Fig. 1, isotherm *b*). This suggests that part of the lipid has formed complexes with the protein that are expelled into the subphase above a surface pressure of 28 mN/m. If such complexes were not formed, all protein molecules should have been expelled in the subphase at the protein collapse pressure, and the rest of the isotherm should have been identical to that of the pure lipid as observed in the case of cytochrome *c* (Lamarche et al., 1988).

Moreover, the results obtained by infrared spectroscopy show that at surface pressures of 10 and 15 mN/m, the penetration of LTP into the DPPG monolayer decreases the order parameter of the lipid acyl chains and also increases the frequency of the bands because of the methylene stretching vibrations (Table 1), whereas no LTP-induced conformational change of the lipid acyl chains is detected at high

surface pressure. Both results show unambiguously that the penetration of LTP into the DPPG monolayer increases the number of *gauche* conformers and thus decreases the conformational order of the phospholipid acyl chains. This last result is characteristic of hydrophobic interactions between a protein or a polypeptide with membranes. The increase in *gauche* conformers has been attributed to the presence of hydrophobic segments of proteins within the hydrophobic core of lipid membranes, these two components interacting strongly together (Taraschi and Mendelsohn, 1979). Taking into account this explanation, it is very likely that at a surface pressure below 20 mN/m, the LTP molecules incorporated into the interfacial film are involved in hydrophobic interactions with the hydrophobic matrix of the lipid monolayer, this interaction being favored by the penetration of LTP within the lipid monolayer. This is consistent with the LTP sequence analysis showing that a large portion of the protein is highly hydrophobic (Désormeaux et al., 1992).

The binding of LTP to DPPG also has a marked effect on the interfacial region of the lipid. Independent of the surface pressure, the protein leads to the appearance of a low frequency component on the carbonyl band, generally attributed in the case of hydrated samples to the carbonyl groups that are hydrogen-bonded to water molecules. Moreover, LTP induces a large increase of the intensity of this band as the proportion of the protein in the mixed film increases, i.e., when surface pressure decreases, suggesting that the protein increases the exposure of the lipid ester groups to the aqueous environment. The observed changes could also be due to hydrogen bonding between the lipid C=O groups and protein residues, such as the OH groups of serine residues that are distributed all along the sequence, or the NH_2 groups of lysyl residues. Early data showed that in dry films of egg-PC/cholesterol mixtures, hydrogen bonding occurs between the cholesterol OH group and the egg-PC carbonyl groups (Zull et al., 1968).

On the other hand, the high frequency component of the carbonyl band shifts to lower frequency as the surface pressure decreases. In addition, the infrared data also indicate that there is a conformational change of the glycerol backbone as a function of the penetration of LTP within the membrane. ATR measurements show that the order parameter for the lipid carbonyl group is -0.17 for pure DPPG and -0.23 for DPPG-LTP complexes, suggesting that the carbonyl groups are more parallel to the interface in the presence of the protein. The shift of the 1740 cm^{-1} component of the carbonyl band can be associated with an increase of the polarity of the environment of the free carbonyl groups, as observed for phospholipids in organic solvent (Blume et al., 1988). Conversely, the binding of extrinsic peptides such as polylysine (Carrier and Pézolet, 1986), polymyxin (Babin et al., 1987), myelin basic protein (Surewicz et al., 1987), and melittin (Lafleur et al., 1991) to liposomes of phosphatidylglycerols leads to a decrease in the dielectric constant of the carbonyl environment. This result further emphasizes the fact that at surface pressures of 10 and 15 mN/m, LTP does not behave as an extrinsic protein and penetrates into the DPPG monolayer. However, the lateral pressure of diacylphosphoglycerol vesicles is not known precisely and thus makes the comparison between monolayer and bilayer data difficult.

The incorporation of LTP into DPPG films at 10 and 15 mN/m is accompanied by an increase in the size of the DPPG domains, as observed by fluorescence microscopy. This effect could be due to the lipid crystal growth in the presence of the protein (Fig. 5). However, if this were the case, there would be an increase in the conformational order of the phospholipid acyl chains as opposed to what is observed by infrared spectroscopy. Therefore the size increase of the solid lipid domains is most likely due to the incorporation of the protein into the domains. This conclusion is supported by fluorescence micrographs using the labeled protein. Fig. 9 *A* shows the presence of the DPPG domains at 15 mN/m from the optical emission of the fluorescein lipid probe. Upon inverting the optical filter to view XRITC-labeled LTP, a diffuse, homogenous fluores-

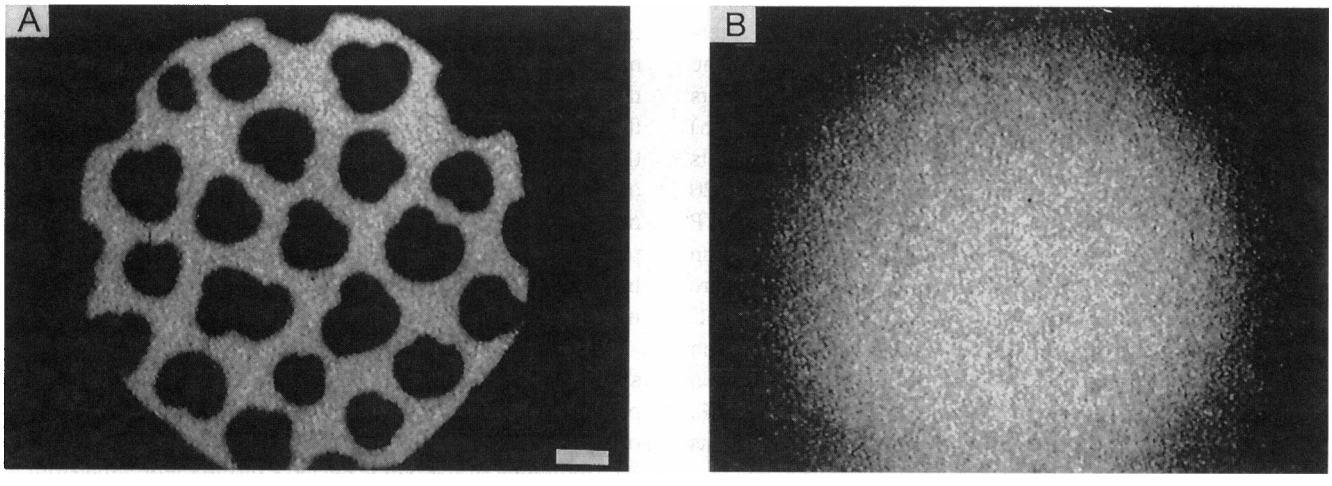

FIGURE 9 Fluorescence micrographs of DPPG domains at 15 mN/m imaged from the optical emission of the fluorescein lipid probe (*A*) and from the optical emission of the sulforhodamine-labeled LTP (*B*).

cent signal could be seen (Fig. 9 *B*). The DPPG domains observed in Fig. 9 *A* cannot be seen in the corresponding Fig. 9 *B* where only the fluorescence originating from the sulforhodamine-labeled LTP can be seen. Conversely, in similar experiments performed with phospholipase A_2, the phospholipid domains were clearly visualized by use of the diffused fluorescence originating from the soluble labeled protein (see Figs. 2 *A* and 3 *A* in Grainger et al., 1989). This difference can be due to the fact that phospholipase A_2 is not incorporated into the phospholipids domains and hydrolyzes only their outer surface (see Fig. 2, *B–F*, in Grainger et al.), whereas part of LTP is incorporated into the solid lipid domains. We can thus conclude from the results obtained at both the molecular and the microscopic levels that the protein is incorporated into both the solid and the liquid lipid domains. Similarly, for mixed monolayers of cytochrome *b* and phospholipids, it has been suggested that the protein was located in the two phases (Heckl et al., 1987).

When LTP is injected at and above 20 mN/m, the compression isotherms as well as the infrared results show that the protein does not penetrate the film. This finding that the penetration of LTP into the lipid film is more important at low surface pressure than in the liquid-condensed phase seems to be quite general, inasmuch as a similar behavior has been reported for the penetration in phospholipid monolayers of cytochrome *b* and *c* (Heckl et al., 1987), cardiotoxin (Bougis et al., 1981), and melittin (Fidelio et al., 1986). Therefore, it seems that the high density and order of lipid monolayers at high surface pressure restricts the penetration of proteins in the lipid monolayer. Nevertheless, fluorescence microscopy shows that at 20 mN/m the protein has an effect on the lipid monolayer even if no expansion of the film is observed. The lipid domains have been perturbed by the presence of the protein, as shown by the appearance of structures protruding from their outer surface. Moreover, the domains obtained when the protein is injected underneath a monolayer at 20 mN/m (Fig. 5 *F*) are very different from those observed at 20 mN/m after compression of the protein-lipid film (Fig. 6 *C*). In fact, much larger domains with no protuberance on their outer surface are then obtained. This suggests that when the protein is injected at and above 20 mN/m, the protein cannot incorporate into the domains directly, but it can further affect the formation of the domains. These results are consistent with the measurements by infrared spectroscopy of the transferred monolayers, which show that at high surface pressure the LTP molecules are bound to the surface of the monolayer and increase the exposure of the lipid ester groups to the aqueous environment.

In addition, protein conformation is an important factor in protein-lipid interactions. Although the high α-helical content of LTP has already been observed for native LTP in aqueous solution (Simorre et al. 1991; Désormeaux et al., 1992) or bound to vesicles of diacylphospholipids or to lysophosphatidylcholine micelles (Désormeaux et al., 1992), our results show that the α-helical content of LTP bound to DPPG monolayers is higher than that of the protein either free in solution or bound to diacylphospholipid vesicles. In addition, the content of α-helix increases with the surface pressure of the mixed monolayers, whereas the mean orientation of the overall helical axis decreases. In fact, the order parameters of the helical segments of LTP suggest that at low surface pressure, the α-helices of the protein are rather parallel to the monolayer plane. However, it is not possible to obtain independent information on the orientation of the individual helical segments of LTP from the current measurements. If we assume that the three-dimensional structure of the LTP at the interface is similar to that obtained in solution, i.e., including four helices not exactly parallel (Gincel et al., 1994), the possibility that one α-helix (H4) is oriented perpendicularly to the membrane while the three others helices are lying parallel to the membrane cannot be excluded. In contrast, at high surface pressure, the α-helices bound to the surface of the monolayer are neither perpendicular nor parallel to the interface but in an oblique orientation.

This study of the interaction of wheat LTP with the anionic lipid DPPG at the air-water interface highlights three main events that depend on the lipid lateral packing pressure: 1) the penetration of at least a part of the LTP molecule into the monolayer of DPPG at low surface pressure (10 and 15 mN/m) and the formation of a LTP-DPPG complex, 2) the transfer of LTP under the lipid monolayer at intermediate surface pressure (20 mN/m), and 3) the expulsion of the LTP-DPPG complex into the subphase above a surface pressure of 28 mN/m. As yet there is no evidence that protein penetration at low surface pressure (step 1) is a prerequisite for lipid transfer activity. However, many LTPs, e.g., phosphatidylcholine-LTP (PC-LTP), phosphatidylionositol-LTP (PL-LTP), or nonspecific-LTP (ns-LTP), exhibit a marked lowering of lipid transfer activity when the membrane fluidity decreases (Helmkamp, 1983). Thus it seems likely that transfer of the phospholipid would require extensive penetration of LTP into the hydrophobic core of the membrane. Our results show that at low surface pressure the protein indeed induces some conformational disorder of the lipid acyl chains. However, they do not reveal clearly how the lipid is bound to the protein.

Recent studies on the structure of plant LTP and LTP-fatty acid complex show that a hydrophobic cleft, formed by residues located in the second half of the protein, is a potential site for the binding of lipids and that this hydrophobic cavity can accommodate only one fatty acyl chain in the all-*trans* conformation, suggesting that the second fatty acyl chain is exposed to solvent (Gincel et al., 1994; Shin et al., 1995; M. Ptak, Centre de Biophysique Moléculaire, Orléans, France, 1994, personal communication). At step 2 (20 mN/m), the infrared studies show that the lipid acyl chain conformation is no longer perturbed by the protein, but protein molecules are still bound to the lipid and hydrophobic interactions exist between LTP and the glycerol moiety of the anionic phospholipid. At first glance, this result suggests that at step 2 the protein is only attached at the surface of the monolayer by hydrophilic interactions

(electrostatic and hydrogen bonds). If this were the case, only the protein would be expulsed in the subphase at step 3, as observed for cytochrome *c* (Lamarche et al., 1988). In order to reconcile the results of the present study and the structural constraints imposed by the hydrophobic cavity of the protein presented above, the three steps occurring during the film compression could be schematically illustrated, as shown in Fig. 10. For all three steps, only one acyl chain of the lipid is bound to the cavity of the protein and its conformation is extended (Shin et al., 1995). The acyl chain conformational disorder observed by infrared spectroscopy for step 1 would be due to the presence of the protein within the lipid domains. As the surface pressure is increased (step 2), the lipid-bound protein is transferred under the lipid monolayer; one acyl chain of DPPG is still in the monolayer while the other is bound to the cavity of the protein. Because the fatty acid bound to the protein is not bent in the hydrophobic protein tunnel (Shin et al., 1995), its conformation should not be different from the one of the fatty acyl chain in the monolayer. To support this model, at step 2 lipid carbonyl groups are involved in hydrogen bonds in agreement with the recently published x-ray structure of a maize LTP-fatty acid complex showing that the fatty acid carboxyl group is involved in hydrogen bonds with Tyr81 (corresponding to Tyr79 in wheat LTP) and two water molecules (Shin et al., 1995). Such a noncovalent acyl chain-mediated anchoring has also been proposed for protein kinase C, an extrinsic membrane protein (Kinnunen et al., 1994). These authors have proposed that a tilt angle of 180° is formed between the two fatty acyl chains of the phospholipid. In our case, such a structural constraint is not necessary because LTP forms an angle of ~60° with respect to the monolayer normal.

One may wonder if this sequence of events mediated by the surface pressure also occurs during the transfer of lipids between bilayer vesicles. It is always a challenging problem to transfer the results obtained with planar monolayers to curved bilayers, inasmuch as it is quite difficult to determine the precise surface pressure in the outer monolayer of bilayer liposomes. However, the LTP-diacylphospholipid interaction model inferred from our monolayer study offers a compelling opportunity to explain the transfer of lipids by ns-LTPs. In fact, for ns-LTP, it is impossible to isolate a stable complex with diacylphospholipids as opposed to specific LTP, even if the transfer obviously means that adsorption of LTP to bilayer interfaces and binding should occur (Kader, 1990). Consequently, a transient collisional complex between donor and acceptor membranes has been postulated to explain lipid transfer by ns-LTPs (Van Amerongen et al. 1989; Billheimer and Gaylor, 1990). This complex is compatible with a shuttle mechanism proposed for the maize LTP (Geldwerth et al., 1991). Our model is congruent with a collisional complex-shuttle mechanism, inasmuch as the transfer of the whole lipid implies a close contact between donor and acceptor membranes to avoid transfer of the second fatty acyl chain in the aqueous solvent. Finally, the dissociation constant determined between plant LTP and fatty acids has a value of about 4 μM for oat nsLTP (Rickers et al. 1984; 1985), which is compatible with a rapid binding-unbinding of the phospholipid molecule between membrane interfaces. Moreover, the improvement of lipid transfer by anionic phospholipids (Petit et al., 1994) could be due to a concomitant increase in protein adsorption and in disorder of the fatty acyl chains, which is observed at low surface pressure in this work. This should facilitate the extraction of one acyl chain of the phospholipid from the bilayer.

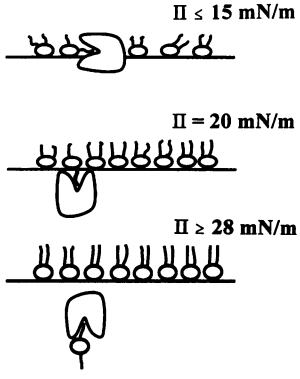

FIGURE 10 Schematic illustration of the different steps involved in the LTP/DPPG interactions

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