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Interactions of Laurdan with phosphatidylcholine liposomes: a high pressure FTIR study

Parkson Lee-Gau Chong [a] and Patrick T.T. Wong [b]

[a] *Department of Biochemistry, Meharry Medical College, Nashville, TN (USA) and* [b] *Steacie Institute for Molecular Sciences, National Research Council of Canada, Ottawa, Ontario (Canada)*

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The interactions of 6-lauroyl-2-dimethylaminonaphthalene (Laurdan) with L-α-dimyristoylphosphatidylcholine (DMPC) have been studied isothermally at 28°C by Fourier-transform infrared spectroscopy (FTIR) at two pH values (6.8 and 3.0) and over the pressure range of 0.001–25 kbar. The results obtained with Laurdan are compared with those previously obtained with 6-propionyl-2-dimethylaminonaphthalene (Prodan) (Chong et al. (1989) Biochemistry 28, 8358–8363). The objective of this study is to delineate the differential interactions of Prodan and Laurdan with lipid membranes. The Laurdan carbonyl and naphthalene vibrational bands as well as the correlation field splitting of the methylene scissoring mode all indicate that in phospholipid model membrane systems, Laurdan behaves differently from Prodan. The data suggest that the chromophore of Laurdan is embedded somewhat deeper in the membrane than that of Prodan. The correlation field splitting pressure suggests that Laurdan causes more perturbation to DMPC vesicles than Prodan. Instead of being relocated to the exterior of the membrane as is the case of Prodan, Laurdan is found to remain in the membrane even when it is partially positively charged at pH 3. Apparently the stabilizing forces come from the strong van der Waals and hydrophobic interactions between the lauroyl chain and its neighboring lipid molecules. Laurdan seems to remain in the membrane at high pressures (up to 25 kbar). Using deuterated DMPC (*d*-DMPC) and deuterated L-α-dipalmitoylphosphatidylcholine (*d*-DPPC), we have demonstrated that, at 1 atm, there is a void space between the lauroyl chain of Laurdan and the acyl chain of the matrix lipid, regardless of the physical state of the matrix lipid. This void space, probably caused by the bulky naphthalene ring, is eventually diminished by elevated pressures.

Introduction

Interaction of small molecules with membranes is an important issue in membrane biology (reviewed in Ref. 1). Hydrophobic fluorescent probes are one type of small molecules of special interest. The use of environmentally sensitive fluorescent probes such as 6-propionyl-2-dimethylaminonaphthalene (Prodan) and 6-lauroyl-2-dimethylaminonaphthalene (Laurdan) (structures shown in Fig. 1) [2] in membrane studies has been increasing in recent years. For example, Prodan fluorescence has been used to investigate the surface nature of lipid bilayers, lipoprotein particles and biological membranes [3–6] and to monitor lipid phase transition and 'membrane fluidity' in both phospholipid model membranes [7,8] and cellular membranes [7,9]. Prodan has also been used to detect the formation of the ethanol-induced interdigitated gel phase of phosphatidylcholine [10]. Laurdan fluorescence, on the other hand, has been used to study the packing of phospholipid tubules [11], the phase transition and the

Correspondence to (present address): P.L.-G. Chong, Department of Biochemistry, Temple University School of Medicine, Philadelphia, PA 19140, USA.

Abbreviations: *d*-DMPC, L-α-dimyristoylphosphatidylcholine deuterated in the acyl chains (d_{54}); *d*-DPPC, L-α-dipalmitoylphosphatidylcholine deuterated in the acyl chains (d_{62}); DMPC, L-α-dimyristoylphosphatidylcholine; DPPC, L-α-dipalmitoylphosphatidylcholine; egg-PC, egg yolk phosphatidylcholine; FTIR, Fourier-transform infrared spectroscopy; Laurdan, 6-lauroyl-2-dimethylaminonaphthalene; Prodan, 6-propionyl-2-dimethylaminonaphthalene.

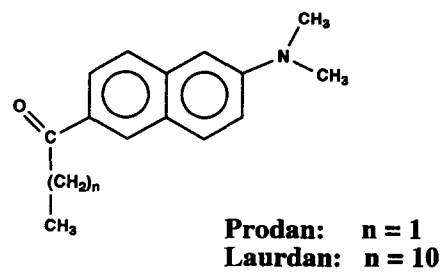

Fig. 1. Structures of Prodan and Laurdan.

phase separation of membrane lipids [12–14], and the membrane surface properties [15]. In addition, Laurdan has been used to detect the lipid fluidity of the sarcoplasmic reticulum Ca-ATPase membranes [16], to compare the fibroblast [17] and red blood cell membranes [18] from patients with Huntington's disease with those from normal subjects, and to monitor the concentration of anesthetics in membranes [14,19].

The present study is to use high pressure Fourier-transform infrared spectroscopy (FTIR) to explore the interactions of Laurdan with lipid bilayers. Pressure is a unique tool for studying intermolecular interactions because pressure can shorten the intermolecular distances, therefore enhancing intermolecular interactions. High pressure FTIR methodology has proven useful in studying interactions of solutes with lipid membranes [20–23]. This methodology has been previously employed to study the interactions of Prodan with L-α-dimyristoylphosphatidylcholine (DMPC) and egg yolk phosphatidylcholine (egg-PC) vesicles [22].

Laurdan differs from Prodan in that Laurdan has a long hydrocarbon chain (11 C) attached to the carbonyl group whereas Prodan has a shorter chain (2 C) (Fig. 1). The chromophores of these two probes are virtually identical. Yet, there are distinctive differences in their behaviors in membranes. Firstly, Laurdan fluorescence can sense the pressure-induced phase transition in egg-PC multilamellar vesicles, whereas Prodan cannot [8]. Secondly, the emission maximum of Prodan fluorescence in L-α-dipalmitoylphosphatidylcholine (DPPC) undergoes a dramatic red shift through the ethanol-induced phase transition from the noninterdigitated gel to the interdigitated gel phase [10]. In contrast, the emission maximum of Laurdan fluorescence remains in the blue region through the phase transition (Zeng and Chong, unpublished results). This suggests that Prodan undergoes relocation in membranes, but Laurdan does not. Thirdly, the intensity ratio of Prodan fluorescence at 435 nm to that at 510 nm ($F435/F510$) has been shown to be a useful index for monitoring the phase transition of lipid membranes [7]. Merlo and Yager [14] used a similar parameter ($F440/F500$) of Laurdan fluorescence to determine the lipid phase transition. However, it is found that the intensity ratio varies with the Prodan concentration in membranes [7], but invariant with the Laurdan concentration [14]. This further suggests that, in membrane systems, Prodan undergoes relocation whereas Laurdan is stabilized in the lipid matrix. Fourthly, Parassasi et al. [13] showed that the fluorescence properties of Laurdan in lipid membranes are independent of the type of the polar headgroup. They suggested that Laurdan is embedded in lipid bilayers deeper than Prodan.

The apparent differences in membrane behaviors between these two probes must lie in the differential interactions of Prodan and Laurdan with lipids. The present study is to delineate such differential interactions by means of high pressure perturbations. Our data reveal the effects of pH and pressure on the location and conformation of Laurdan in DMPC vesicles as well as the effect of Laurdan on lipid packing. The results will form the bases for future studies of solute partitioning in membranes at high pressures.

Materials and Methods

Preparation of lipid membranes

DMPC, DPPC, deuterated DMPC-d_{54} (d-DMPC) and deuterated DPPC-d_{62} (d-DPPC) were purchased from Avanti Polar Lipids (Alabaster, AL). Laurdan was obtained from Molecular Probes (Eugene, OR). Lipids and Laurdan were co-dissolved in chloroform in an Eppendorf vial and then dried with nitrogen. The dried sample was mixed with a trace amount of D_2O in order to remove the tightly bound chloroform from the carbonyl groups of the lipids. The mixture was then lyophilized overnight. The lyophilized lipid/probe mixture was dispersed by vortexing in a Tris buffer made with D_2O. The concentrations of Laurdan and lipids were calculated from weight determinations.

Determination of pK_a for Prodan and Laurdan

Prodan is marginally soluble in water and Laurdan is insoluble. In order to determine the pK_a value, we have converted the probes to their salt forms. 35 mg of Prodan was dissolved in 1 ml of chloroform and then mixed with 1 ml of 2 M HCl. The mixture was stirred overnight at room temperature. The aqueous layer was separated from the chloroform layer and evaporated to dryness to obtain the HCl salt of Prodan (37 mg). This salt was then dissolved in methanol and titrated with CH_3ONa. A direct titration of Prodan salt with NaOH was not used because precipitation appeared during the titration. The pK_a value of Prodan was determined to be 1.7 by our method. The pK_a of Laurdan should be close to this value because of the structural similarity between Prodan and Laurdan. Note that although this experimentally determined pK_a value is lower than the value (4.5) previously estimated [22], our conclusion pertaining to the effect of pH on Prodan IR data [22] remains valid, since the conclusion was made at a qualitative level.

Measurement of IR spectra at high pressures

Small amounts of the well-mixed lipid/Laurdan dispersions were placed, together with powdered α-quartz, in a 0.37-mm diameter hole in a 0.23-mm thick stainless-steel gasket mounted on a diamond anvil cell, as described previously [24]. Pressure was determined from the 695 cm^{-1} phonon band of α-quartz. The frequency of this band shifts as the pressure varies.

The pressures were calculated according to the method described previously [24,25]. Infrared spectra were measured at 28°C on a Bomem Model Michelson 110 Fourier-transform spectrophotometer with a liquid nitrogen cooled mercury-cadmium-telluride detector. For each spectrum, 300 scans were performed at a spectral resolution of 4 cm^{-1}. The vibrational frequencies were obtained from third-order derivative spectra. All of the data analysis including the determination of the correlation field splitting pressure was made with the computer program developed by the vibrational spectroscopy laboratory at the National Research Council of Canada.

Results and Discussion

C=O vibrational bands

Fig. 2A shows the stacked contour plots of the infrared spectra in the C=O stretching region for Laurdan dispersed in D_2O. The bottom curve in Fig. 2A shows that the C=O stretching band of Laurdan in D_2O at 1 kbar is at about 1662 cm^{-1}. This frequency is much lower than that of Prodan in D_2O (1674 cm^{-1}; [22]). The bottom curve in Fig. 2B shows the C=O vibrational bands in the mixture of Laurdan (11 mol%) in DMPC at pH 6.8 and at 0.7 kbar. The C=O band of Laurdan (1676 cm^{-1}) is broad and barely visible whereas the C=O band of DMPC (1730 cm^{-1}) is sharp and strong. This suggests that the C=O group of Laurdan is embedded in the lipid matrix at pH 6.8 [20–22]. Based on the emission maximum of Laurdan fluorescence, Sumbilla and Lakowicz [18] previously suggested that Laurdan is embedded in the membrane and not completely exposed to the aqueous phase. Here we provide more direct evidence showing the location of Laurdan in membranes. The Laurdan concentration ultilized in the current study is 11 mol%. If probe aggregation occurs at this concentration, a sharp and strong C=O peak for Laurdan should be observed. This is apparently not the case (Fig. 2).

The pK_a of the amino group in Prodan and Laurdan is about 1.7, as mentioned in Materials and Methods. Thus, at pH 3, about 5% of Laurdan molecules are protonated and positively charged. As a result, the C=O band of Laurdan becomes more noticeable at pH 3.0 (Fig. 2C) than at pH 6.8 (Fig. 2B). This result is attributed to the vertical motion of positively charged Laurdan molecules toward the lipid/water interfacial region when the pH is changed from 6.8 to 3.0, since it is known that the charged molecule has lower membrane permeability than the neutral species. However, the Laurdan C=O band at pH 3.0 (Fig. 2C) is still weak, as compared to the strong C=O band of Prodan in DMPC at the same probe concentration (11 mol%) and at the same pH [22]. It is possible that when Laurdan is positively charged, Laurdan moves toward the membrane surface, but is still within the membrane. In contrast, at pH 3, the protonated Prodan is situated outside the membrane [22]. Note that even 5% of Prodan molecules are protonated and situated outside the membrane; they will contribute significantly to the increase of the intensity at the C=O absorption band.

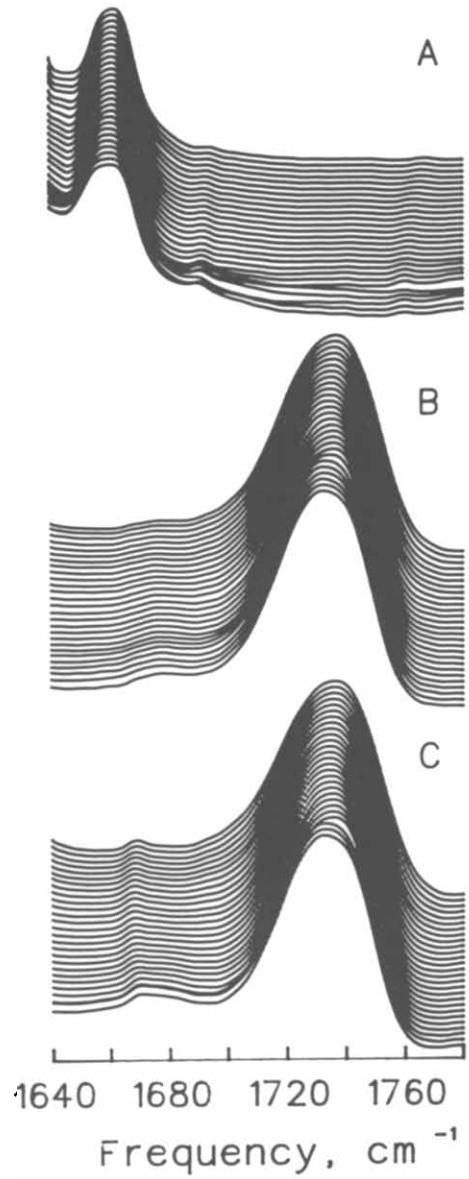

Fig. 2. Stacked contour plots of the infrared spectra in the 1640–1780 cm^{-1} region. (A) Pure Laurdan in D_2O (from bottom to top: 1 kbar to 15.6 kbar); (B) 11 mol% Laurdan in DMPC at pH 6.8 (from bottom to top: 0.7 kbar to 17.1 kbar); (C) 11 mol% Laurdan in DMPC at pH 3.0 (from bottom to top: 0.8 kbar to 16.7 kbar).

The difference in probe behavior is also obvious from the frequency data. At low pressures (< 1 kbar), the frequency of the Laurdan C=O stretching band (1672–1674 cm^{-1}, Figs. 2C and 3) in DMPC at pH 3.0 is much higher than the C=O frequency of Laurdan in D_2O (1662 cm^{-1}, Figs. 2A and 3). This suggests that

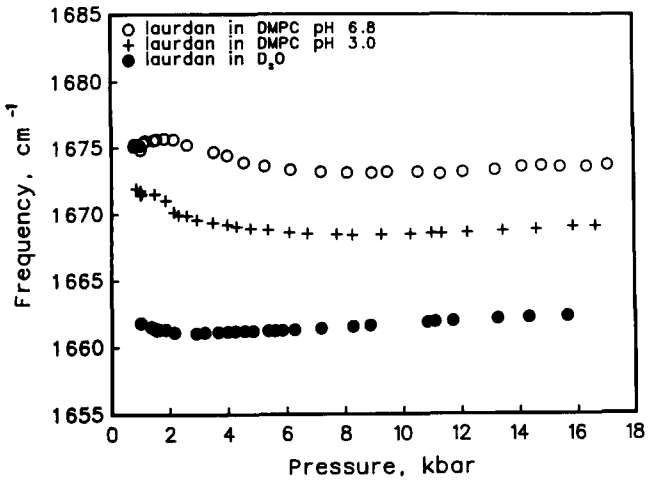

Fig. 3. Pressure dependence of the Laurdan C=O stretching frequency in the sample of pure Laurdan in D_2O (●), 11 mol% Laurdan in DMPC at pH 6.8 (○), and 11 mol% Laurdan in DMPC at pH 3.0 (+).

Laurdan is inside the membrane at pH 3. In a previous study, we have shown that the C=O frequency of Prodan in DMPC is lowered to a value almost identical to the C=O frequency of Prodan in D_2O when the pH is changed from 6.8 to 3.0 [22]. This result was interpreted as Prodan being relocated to a site outside the membrane [22].

The difference in probe behavior is further evident in the pressure dependence of the C=O vibrational frequency. Here two factors should be considered. Firstly, the C=O frequency should increase with increasing pressure as a result of compression. Secondly, the C=O frequency should decrease due to the pressure-induced strengthening of the hydrogen bond formed between the C=O group of Laurdan and its neighboring molecules (e.g., water). In the case of Prodan, these factors offset each other, thus the C=O frequency of Prodan in DMPC is almost invariant with pressure [22]. However, in the case of Laurdan in DMPC at pH 3, a steady decrease in the C=O frequency is seen in the pressure range of 0.001–7.0 kbar (Fig. 3, +). This may be due to the pressure-induced strengthening of the hydrogen bonds. At pH 6.8, the C=O frequency of Laurdan initially increases with increasing pressure until 2 kbar, then is followed by a steady decrease (Fig. 3, ○). As mentioned earlier, at pH 6.8, Laurdan is located somewhat deeper in the lipid bilayer than at pH 3.0. The initial increase in the C=O frequency at pH 6.8 is probably due to the effect of compression on the C=O bond. The subsequent decrease could be due to the pressure-induced strengthening of the hydrogen bonds; here it is assumed that the association of the C=O group of Laurdan with water is not affected by pH.

It should be remarked that the changes in infrared spectra due to the changes in pH can only be partially explained by electron delocalization. When the amino group of Prodan or Laurdan is positively charged, the vibrational frequency of the probe C=O band is weakened due to the electron transfer through the conjugated system toward the amino group. This would result in a lower C=O vibrational frequency at pH 3, as compared to the frequency at pH 6.8. This is indeed the case as shown in Fig. 3 and in Ref. 22. However, the Laurdan C=O stretching frequency in DMPC at pH 3 does not drop to the C=O frequency of Laurdan in D_2O (Fig. 3). In contrast, the Prodan C=O vibrational frequency in DMPC at pH 3 drops to a level almost identical to the C=O frequency of Prodan in D_2O [22]. These frequency changes cannot be interpreted simply by electron delocalization. But, they can be satisfactorily explained by electron delocalization plus the concept of probe relocation, as described earlier.

The difference in probe behavior can be explained as follows. It is known that the membrane/water partition coefficient is increased by a factor of 2–3 for each additional methylene unit in the solute molecule [26]. Thus, the long hydrocarbon tail (11 C) of Laurdan can provide strong van der Waals and hydrophobic interactions with its neighboring lipid molecules. These attractive forces are strong and cannot be counterbalanced by the increase in electrostatic repulsive interactions due to the partially positive charge on the probe at pH 3.0. In contrast, the hydrocarbon chain (2 C) next to the Prodan C=O group is too short to provide strong van der Waals and hydrophobic interactions with neighboring lipid molecules. This explains why Prodan undergoes relocation in membranes, whereas Laurdan does not (Ref. 7, Zeng and Chong, unpublished results). Similar assertions have been previously proposed to explain the chain-length dependence of the interactions of n-alkanes and n-alcohols with lipid bilayers (e.g., Ref. 27).

Correlation field splitting of the methylene scissoring mode in DMPC

In order to assess which probe, Laurdan or Prodan, causes a greater disordering effect on the packing of matrix lipids, we have determined the correlation field splitting pressure, P_s, of the DMPC methylene scissoring mode in the presence of the probes. The pressure-induced correlation field splitting of the methylene scissoring mode has proven useful in characterizing the interchain packing [28,29]. The tighter the packing, the lower the P_s is. Figs. 4A and 4B show the stacked contour plots of the infrared spectra in the methylene scissoring mode region (1400–1525 cm^{-1}). Figs. 5A and 5B show the correlation field splitting of the DMPC methylene scissoring mode in the presence of Laurdan (11 mol%) at pH 6.8 and at pH 3.0, respectively. The P_s for DMPC containing Laurdan (11 mol%) at pH 6.8 is 4.6 kbar. This value is much higher than

the P_s (3.9 kbar) for DMPC containing Prodan (11 mol%) (4% by weight) at the same pH [22]. This result suggests that Laurdan causes more perturbation to DMPC vesicles than Prodan. This can be taken to indicate that the chromophore of Laurdan is embedded deeper in the lipid bilayer than the chromophore of Prodan since the perturbation should mainly arise from the bulky chromophore, rather than from the methylene units of the lauroyl chain. This notion seems to agree with the conclusion derived from the fluorescence data of Parassasi et al. [13] who suggested a deeper penetration into the lipid bilayer by Laurdan because the generalized fluorescence polarization of Laurdan was found to be invariant with the type of lipid headgroup.

At pH 3.0, the P_s for DMPC containing Laurdan (11 mol%) is determined to be at 4.8 kbar. This value is close to that at pH 6.8, suggesting a similar perturbation at pH 3. This result is consistent with the C=O data (Fig. 3) which infers that at pH 3, Laurdan does not move out of the membrane.

Naphthalene rings

The naphthalene ring of Laurdan can be detected from the 1510–1520 cm^{-1} region. Fig. 4A shows that for Laurdan (11 mol%) in DMPC at pH 6.8, the naphthalene band is barely noticeable, a situation similar to Prodan (11 mol%) in DMPC at pH 6.8 [22]. This is thought to be the result of the ring being embedded in the lipid matrix [22]. In the case of Prodan, the naphthalene band becomes sharp and discernable when the pH is lowered to 3.0 [22]. This was interpreted as the naphthalene of Prodan moving out of the membrane due to the partial positive charge of the amino group at pH 3.0. This is apparently not the case for Laurdan. The naphthalene band of Laurdan (11 mol%) in DMPC remains weak and broad at pH 3.0, although the band is somewhat more noticeable (Fig. 4B). The naphthalene data, together with the C=O and the correlation field splitting data, strongly suggest that Laurdan tends to remain in the lipid matrix, even when it is partially positively charged and under high pressures.

Laurdan in d-DMPC and in d-DPPC

It can be postulated that the polymethylene chain of Laurdan has its own conformational changes when the matrix lipids undergo phase transition. Since the chromophore of Laurdan is covalently linked to the polymethylene tail, the orientation of the chromophore with respect to the membrane surface may be altered through the phase transition of the matrix lipid. If so, the interactions between the excited state dipole and the 'solvent' dipole may be significantly altered, as suggested by Parasassi et al. [12]. The conformational change in the polymethylene region of the Laurdan

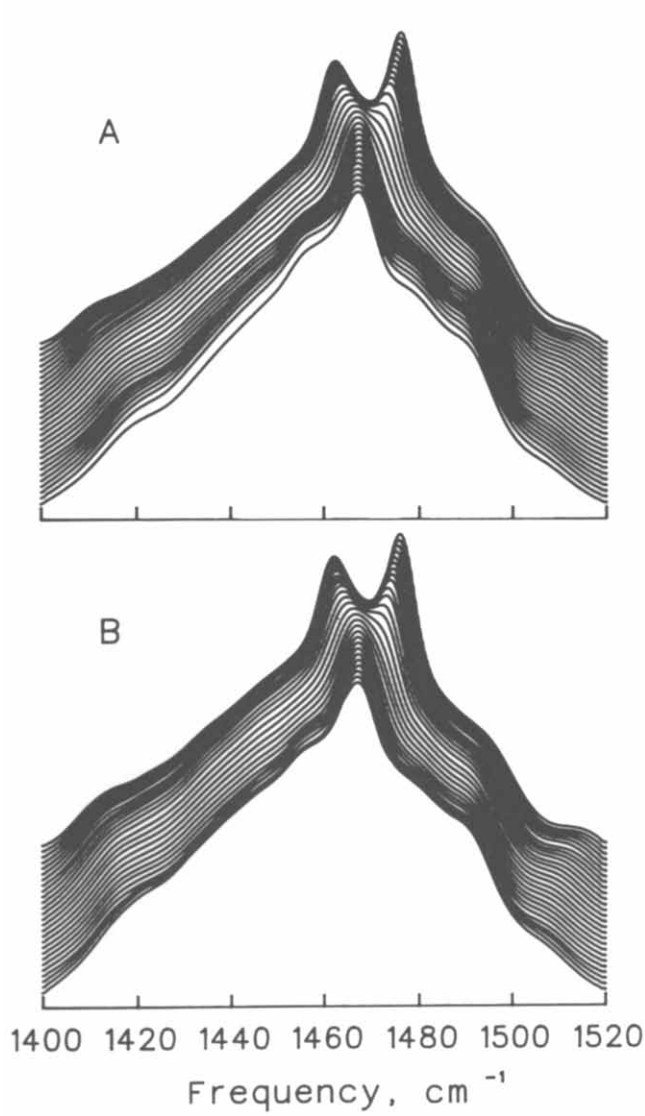

Fig. 4. Stacked contour plots of the infrared spectra in the 1400–1520 cm^{-1} region. (A) 11 mol% Laurdan in DMPC at pH 6.8 (from bottom to top: 0.7 kbar to 17.1 kbar); (B) 11 mol% Laurdan in DMPC at pH 3.0 (from bottom to top: 0.8 kbar to 16.7 kbar).

molecule can thus contribute to the changes in Laurdan fluorescence by either temperature or pressure.

In order to test this postulation, we have measured the infrared spectra of Laurdan in deuterated DMPC (*d*-DMPC) and in deuterated DPPC (*d*-DPPC). Special attention is focused on the methylene symmetric stretching mode (2850–2852 cm^{-1}). *d*-DMPC and *d*-DPPC were used to move the methylene symmetric stretching bands of lipids to other frequency regions. This allows the methylene symmetric stretching mode of Laurdan to be observed.

As shown in Fig. 6 (○), the methylene stretching frequency of Laurdan in *d*-DMPC at 1 atm is at 2851 cm^{-1}. This frequency decreases slightly with pressure until 3 kbar. Beyond 3 kbar, the vibrational frequency of the methylene stretching mode increases with pressure in a linear manner. For pure lipids, an increase in

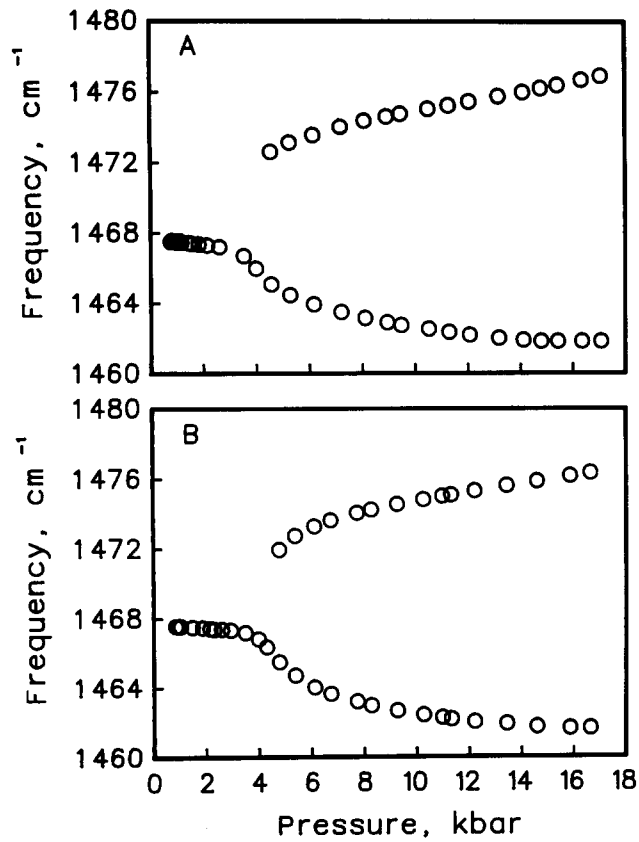

Fig. 5. Pressure-induced correlation field splitting of the methylene scissoring mode in the sample of (A) 11 mol% Laurdan in DMPC at pH 6.8, and (B) 11 mol% Laurdan in DMPC at pH 3.0.

the methylene stretching frequency with increasing pressure is observed *only when* the lipid is in the ordered gel state. Fig. 6 (○) thus indicates that the lauroyl chain undergoes a disordering-ordering transition in *d*-DMPC matrix at about 3 kbar. However, this pressure is much higher than the liquid crystalline-to-gel phase transition pressure of pure DMPC (0.15 kbar at 28°C, [30]), and higher than that of DMPC in the presence of 11 mol% Laurdan (about 1.5 kbar, data not shown). This result suggests that there exists a void space between the lauroyl chain and the lipid at pressures < 3 kbar. This void space becomes smaller as the pressure is increased. Above 3 kbar, the void space is no longer available; then, the lauroyl chain becomes ordered. These results are similar to those obtained with hexadecane in DMPC [23]. However, the conformationally disordered structure of intercalated hexadecane in DMPC is maintained until the pressure reaches 2.4 kbar at 28°C [23] whereas, at the same temperature, such a critical pressure occurs at 3 kbar for Laurdan in DMPC. This difference (2.4 kbar vs. 3 kbar) can be attributed to the membrane perturbation caused by the intercalation of the bulky naphthalene moiety of Laurdan in the membrane. This assertion is consistent with the conclusion made earlier that Laurdan is embedded in the lipid matrix.

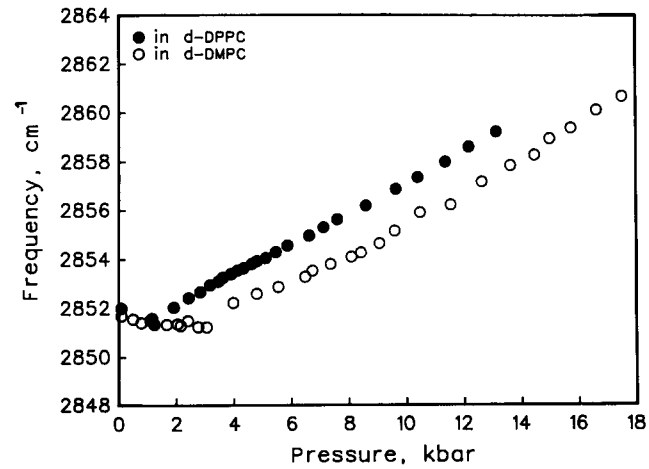

Fig. 6. Effects of pressure on the vibrational frequency of the methylene symmetric stretching mode: 11 mol% Laurdan in *d*-DMPC at pH 6.8 (○) and 11 mol% Laurdan in *d*-DPPC at pH 6.8 (●).

In order to test whether the conformation of the lauroyl chain varies with the type of matrix lipid, we have used *d*-DPPC to replace *d*-DMPC as the matrix lipid. Fig. 6 (dark circles) shows the results obtained with Laurdan (11 mol%) in *d*-DPPC at pH 6.8. The methylene stretching frequency decreases with pressure until 1 kbar, indicating that the lauroyl chain in *d*-DPPC is turned into an ordered state at 1 kbar. This value is lower than that observed with *d*-DMPC, indicating that the conformation of the lauroyl chain varies with the matrix lipids. However, at 28°C and 1 atm, pure DPPC is already in its gel state. The results of Fig. 6 thus demonstrate that, at 1 atm, there is a void space between the lauroyl chain of Laurdan and the acyl chain of the matrix lipid, even in the gel state of the matrix lipid. This void space, probably caused by the bulky naphthalene ring, is eventually diminished by compression at high pressures.

Final remarks

The probe concentration ultilized in this study is much higher than that normally used in fluorescence experiments. However, our goal is not to compare the fluorescence data with the IR data; instead, we compare the IR data obtained from Laurdan with the IR data of Prodan previously published [22]. This comparison was made at the same probe concentration, at the same pHs and in the same type of matrix lipid. Under these conditions, the observed differences in the IR data between these two probes are significant and can reflect the differential interactions of these probes with membranes. In addition, the present study reveals the location and conformation of Laurdan in lipid bilayers at high pressures. This information will form the base for our future use of Laurdan in studying membrane properties at high pressures.

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