Structure and Dynamics of Dimyristoylphosphatidic Acid/Calcium Complexes by ^2H NMR, Infrared, and Raman Spectroscopies and Small-Angle X-ray Diffraction†

Gaëtan Laroche,‡ Erick J. Dufourc,§ Jean Dufourcq,§ and Michel Pézolet*,‡

Centre de Recherche en Sciences et Ingénierie des Macromolécules, Département de Chimie, Université Laval, Cité Universitaire, Québec, Canada G1K 7P4, and Centre de Recherche Paul Pascal/CNRS, Avenue Schweitzer, F-33600 Pessac, France

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ABSTRACT: The structural and dynamic properties of complexes of dimyristoylphosphatidic acid (DMPA) and calcium ions have been characterized by ^2H NMR, Raman, and infrared spectroscopies and small-angle X-ray diffraction. All techniques used show that these complexes do not undergo a cooperative thermotropic phase transition. Small-angle X-ray diffraction unambiguously demonstrates that the structure of the lipid molecules of the DMPA/Ca^{2+} complexes remains lamellar even at a temperature as high as 85 °C. Raman results indicate that within this temperature range, only a few trans–gauche isomerizations of the C–C bonds of the phospholipid acyl chains arise in this system. The ^2H NMR spectra indicate that the DMPA chains are highly motionally restricted up to 65 °C and that higher temperatures might activate some low-frequency overall motions of entire lamellar domains. Small-angle X-ray scattering and ^2H NMR spectroscopy of ^2H_2O also show that the interaction of calcium with DMPA promotes an important dehydration of the lipid assembly, even though the latter technique clearly demonstrates that some water molecules remain strongly bond in the DMPA/Ca^{2+} complexes. The carbonyl stretching mode region of the infrared spectrum of DMPA/Ca^{2+} complexes suggests that these water molecules are trapped near the interfacial region of the lipid membrane and are hydrogen bonded with the carbonyl groups of the lipid. Finally, comparison of the phosphate stretching mode region of the infrared spectra of complexes of DMPA with calcium ions with those of model compounds provides strong evidence that calcium ions bind to both charges of the phosphate group of DMPA and form bridges between adjacent bilayers.

The binding of calcium ions to phospholipids occurs in several physiological processes such as nerve excitation and membrane fusion (Poste & Allison, 1973; Cullis & de Kruijff, 1979; Leventis et al., 1986; Papahadjopoulos et al., 1974; Düzgünes et al., 1984; Hammoudah et al., 1979). It is also well-known that Ca^{2+} ions can induce drastic reorganization of the phospholipid bilayer structure toward nonbilayer ones (Van Venetië & Verkleij, 1981; Hope & Cullis, 1980; Verkleij et al., 1982) or to "cochleate lipid cylinders" (Papahadjopoulos et al., 1975; Portis et al., 1979). Even though these structures have been extensively studied, the effect of calcium on the dynamic and conformational properties of the different parts of phospholipid molecules is still not fully understood.

Most studies on the interaction of calcium ions with acidic phospholipids have been performed on phosphatidylserines (PS).[1] Mattai et al. (1989) have concluded from ^{31}P NMR spectroscopy measurements that calcium binds to phosphatidylserine and leads to the immobilization of the phosphate group of this lipid. In addition, infrared spectroscopy (Dluhy et al., 1983; Casal et al., 1987a,b,c) failed to detect any phase transition when calcium is bound to phosphatidylserine and showed that the entire polar head group of the lipid is immobilized.

Only a few experiments have been done on the effect of calcium ions on anionic phospholipids other than PS. Differential scanning calorimetry has shown that the gel-to-fluid phase transition temperature of didodecanoylphosphatidylglycerol increases markedly in the presence of calcium ions (Verkleij et al., 1979) while in the case of DMPA, no observable transition is even detected between 0 and 100 °C for excess Ca^{2+} (Van Dijck et al., 1978; Liao & Prestegard, 1981; Graham et al., 1985; Kouaouci et al., 1985). For both lipids, the addition of excess Ca^{2+} results in a disorganization of the liposomal bilayers and a conversion into highly packed cylindrical lamellar structures which precipitate in water (Verkleij et al., 1979; Van Dijck et al., 1978).

In order to further investigate the effect of calcium ions on the phosphate group, the interfacial region, and the acyl chain of the DMPA molecules, we have used in the current study several complementary physical techniques, namely, ^2H NMR, Raman, and infrared spectroscopies and small-angle X-ray diffraction.

NMR spectroscopy is particularly useful to monitor several important parameters of phospholipid systems. For example, ^2H NMR spectroscopy of phospholipids with a perdeuterated acyl chain gives information about dynamic and conformational properties of phospholipid systems (Davis, 1983; Dufourc et al., 1984; Huschilt et al., 1985) since the width and the shape of the spectra provide information about the morphology of the phases adopted by phospholipid molecules. Moreover, in ^2H_2O-containing systems, ^2H NMR spectroscopy can be used

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* To whom correspondence should be addressed.

‡ Université Laval.

§ Centre de Recherche Paul Pascal/CNRS.

[1] Abbreviations: NMR, nuclear magnetic resonance; DMPA, dimyristoylphosphatidic acid; PS, phosphatidylserine; [*sn*-2-$^2H_{27}$]DMPA, 1-myristoyl-2-perdeuteriomyristoyl-*sn*-glycero-3-phosphoric acid; DHPA, dihexadecylphosphatidic acid; MMP, monomethyl phosphate; DOPA, dioleoylphosphatidic acid; PG, phosphatidylglycerol; PA, phosphatidic acid.

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to characterize the structure and dynamics of water at the interface.

On the other hand, Raman and infrared spectroscopies are particularly well suited for the determination of the conformation and packing of the different parts of the phospholipid molecules. Raman spectroscopy is quite effective to probe the conformation and packing of the acyl chains of phospholipids (Gaber & Peticolas, 1977; Laroche et al., 1988; O'Leary & Levin., 1984; Lafleur et al., 1987; Carrier & Pézolet, 1984, 1986) while infrared spectroscopy provides valuable information on the structure of the polar head group and interfacial regions (Wong et al., 1989; Mantsch et al., 1989; Babin et al., 1987).

Even though these spectroscopic techniques may have spectral signatures of the type of structure adopted by phospholipids, X-ray diffraction is one of the few techniques that can confirm unambiguously the existence of a given type of structure in phospholipid membranes. Several studies have already shown that the X-ray diffraction pattern of phospholipid systems is the fingerprint of the phase adopted by the lipid and allows the quantitative determination of the structural parameters of these phases (Tardieu & Luzzati, 1970; Shyamsunder et al., 1988; Seddon et al., 1984; Lewis et al., 1989; Tate & Gruner, 1989). By combining these techniques, one may expect to obtain a comprehensive description of the structure and dynamics of Ca^{2+}/DMPA complexes.

Materials and Methods

Materials. The disodium salt of dimyristoylphosphatidic acid (DMPA) and that of dimyristoylphosphatidic acid with a perdeuterated *sn*-2 chain ([*sn*-2-$^2H_{27}$]DMPA) were obtained from Avanti Polar Lipids (Birmingham, AL). Dihexadecylphosphatidic acid (DHPA) was obtained from Medmark (Munich, Germany) while the dicyclohexylammonium salt of monomethyl phosphate was purchased from Sigma Chemical Co. (St. Louis, MO). Deuterium-depleted water (^1H_2O) and heavy water (^2H_2O) were obtained from Aldrich Chemicals (Strasbourg, France) and CEA (Saclay, France), respectively. All materials were used without further purification.

NMR Experiments. Aqueous dispersions of [*sn*-2-$^2H_{27}$]-DMPA were prepared by mixing appropriate amounts of the lipid in deuterium-depleted water at pH 6.5. Samples were then heated to 65 °C for 10 min, vortexed, and cooled down below the gel-to-fluid phase transition. This cycle was repeated several times in order to ensure a good homogeneity of the dispersions. The pH of the samples was measured and adjusted to 6.5 by dropwise addition of concentrated NaOH, if necessary, and the samples were transferred into 10-mm-diameter NMR tubes. Samples containing calcium ions were prepared by dispersing the dry lipid in an appropriate amount of a 70 mM $CaCl_2$ solution in order to obtain a calcium to lipid mixing molar ratio of **3**. They were then heated to 80 °C for 10 min and cooled down below 20 °C several times. The pH of the dispersion was adjusted between each cycle until no pH variation was observed. For all experiments, the final lipid concentration was 5% by weight. To study the structure of water at the interface, H_2O was replaced by ^2H_2O, and the above-mentioned procedure was followed except that the sample tubes were only 4 mm in diameter. Deuterium spectra were recorded on a Bruker MSL 200 NMR spectrometer operating at 30.7 MHz by mean of a quadrupolar echo sequence. Quadrature detection was utilized, and the temperature was regulated to ±1 °C. Samples were allowed to equilibrate at a given temperature for at least 30 min prior to recording the NMR signal. Typical experimental parameters were as follows: $\pi/2$ pulse length of 5.25 μs; delay between the pulses to form the echo of 20 μs; spectral window of 500 kHz; and a recycle time of 1.5–3 s. ^{31}P measurements were performed on the same spectrometer operating at 81 MHz by means of the Hahn-echo pulse sequence (Rance & Byrd, 1983). Typical experimental parameters were the following: $\pi/2$ pulse length of 4 μs; delay between the pulses to form the echo of 20 μs; spectral window of 50 kHz; and recycle time of 6 s. Data were treated on a VAX/VMS 8600 computer.

Raman Experiments. Dispersions of DMPA in the absence and presence of Ca^{2+} ions were prepared as for the NMR experiments, but the final lipid concentration was 10% by weight instead of 5%. Samples were then transferred in glass capillary tubes and spun down in a haematocrit centrifuge. Raman spectra were obtained from the white pellet. The Raman spectrometer used in this work is described elsewhere (Savoie et al., 1979; Pézolet et al., 1983). Samples were excited with the 514.5-nm line of a Spectra Physics Model 2020 argon ion laser; the laser power at the sample was approximately 150 mW. Spectra were recorded digitally with an integrating period of 2 s.

Infrared Experiments. Dispersions for infrared spectroscopy were prepared as for NMR experiments (5% lipid by weight) in ^2H_2O or in H_2O in order to minimize the solvent spectral contribution in the carbonyl or phosphate stretching mode regions, respectively. Spectra were recorded with a Bomem DA3-O2 Fourier-transform infrared spectrophotometer with a narrow-band mercury–cadmium–telluride detector and a germanium-coated KBr beamsplitter. A total of 1000 scans were routinely recorded with a maximal optical retardation of 0.5 cm, co-added, triangularly-apodized, and Fourier-transformed to yield a resolution of 2 cm^{-1}. For pure DMPA dispersions, samples were placed between two BaF_2 windows, and transmission spectra were recorded. For complexes with calcium, spectra were obtained by internal reflectance on a thallium–bromide–iodide (KRS-5) prism in order to eliminate artifacts caused by the inhomogeneity of the sample, such as broadening of the features in the C–H stretching mode region. In this case, the white precipitate was spread over the prism and covered with the supernatant solution.

Spectra in the carbonyl stretching mode region were corrected for the combination band of ^2H_2O by subtracting a straight base line between 1650 and 1780 cm^{-1}, while those in the phosphate stretching mode region were corrected for the BaF_2 or KRS-5 and water absorption by subtracting an appropriate polynomial function.

Small-Angle X-ray Experiments. Samples used for Raman experiments were transferred into X-ray thin-wall glass capillary tubes after recording the Raman spectra; capillaries were then centrifuged and sealed. X-ray measurements were performed on a Rigaku/Rotaflex X-ray diffractometer (Model RU-200 BH) equipped with a rotating anode, using the nickel-filtered Cu Kα line (λ = 1.5418 Å) focused by two 0.65-mm-diameter pinholes; the power of the beam was 8 kW. The detector was placed at approximately 20 cm from the sample, this space being kept under vacuum. Diffraction patterns were recorded digitally with a scan rate of 0.01°/6 s using a scintillation detector by integrating a circular arc within 10° of a line perpendicular to the long axis of the X-ray beam cross section. Samples were placed in a copper sample holder thermoregulated to ±1 °C.

Results

Structure and Dynamics of the Acyl Chains. Figure 1 (left) shows the ^2H NMR spectra of pure [*sn*-2-$^2H_{27}$]DMPA. As can be seen, the ^2H NMR spectra of labeled DMPA are quite

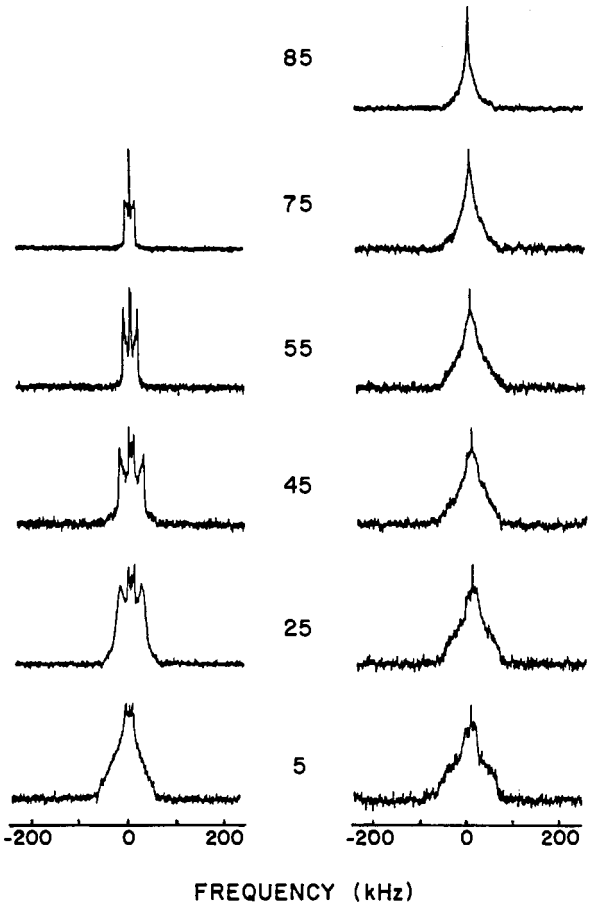

FIGURE 1: Temperature dependence of the ^2H NMR powder spectra of [*sn*-2-$^2H_{27}$]DMPA in the absence (left) and presence of calcium ions (right). Temperature in degrees centigrade is indicated near each spectrum. Each spectrum is the sum of approximately 5000 scans.

sensitive to the gel-to-fluid phase transition of this lipid. The gel phase powder pattern spectra (bottom) span a broad range of frequency of approximately ±65 kHz while the fluid phase spectra (top) cover only a frequency range of approximately ±35 kHz about the deuterium Larmor frequency. Furthermore, the fluid phase spectrum is characterized by a narrow methyl group component with a splitting of 2 kHz, measured between the so-called 90° orientations and several 90° edges which characterize the motional averaging of labeled methylene groups from the center of the bilayer to the phospholipid polar head group. For labeled positions near the polar head group (e.g., positions 3–9), the motional averaging is approximately the same, and this results in unresolved peaks forming the so-called "plateau" region (Laroche et al., 1990). On the other hand, the gel phase spectrum of [*sn*-2-$^2H_{27}$]-DMPA (bottom) exhibits a methyl group component of approximately 16 kHz while the methylene groups give rise to a broad spectrum. The differences observed between these two types of spectra allow the investigation of the thermotropic phase behavior of a phospholipid system and the determination of the phase transition temperature. In the case of pure DMPA, the above-mentioned changes in the powder pattern spectra occur mainly between 45 and 50 °C (Laroche et al., 1990).

Figure 1 (right) shows that the addition of excess of calcium ions to DMPA strongly affects the temperature dependence of the ^2H NMR spectra of the lipid. The spectrum of DMPA/Ca^{2+} complexes at low temperature is very broad, showing that the movement of the lipid acyl chains is highly restricted. When the temperature of the system is increased

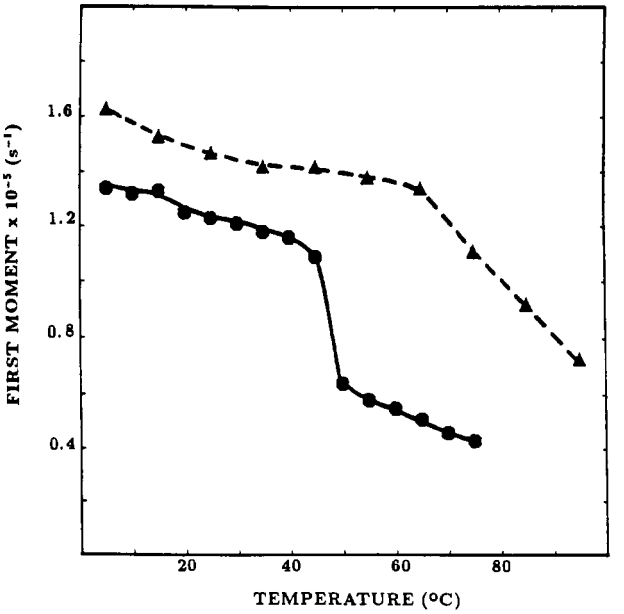

FIGURE 2: Temperature dependence of the first moment of [*sn*-2-$^2H_{27}$]DMPA ^2H NMR spectra in the absence (●) and presence (▲) of calcium ions.

above 45 °C, the axially symmetric powder pattern characteristic of the lipid fluid phase is not observed. Instead, the features of the gel phase are still present but become progressively narrower as the temperature is increased. At 85 °C, a narrow "isotropic" line dominates the spectrum, but the broad edges from the gel phase are still detected. Therefore, these results show that in the presence of calcium ions, DMPA does not undergo the gel-to-fluid phase transition but either symmetry or dynamic changes occur at high temperature.

In order to quantify the effect of calcium on the thermotropic behavior of DMPA, ^2H NMR spectral moments were calculated (Bloom et al., 1978; Davis, 1979). The first spectral moment, M_1, calculated with respect to the middle of the spectra (zero frequency) from the absolute value of the frequencies, allows an estimation of the orientational order parameter for a given deuterium-labeled carbon position. Since in the current study first spectral moments were calculated from spectra originating from perdeuterated acyl chains, they will only provide a measure of the overall chain order parameter. Figure 2 displays the temperature dependence of M_1 for [*sn*-2-$^2H_{27}$]DMPA. As seen, the first spectral moment of pure DMPA dispersion spectra decreases with temperature, and the gel-to-fluid phase transition occurs at 47 °C, in good agreement with previous Raman (Laroche et al., 1988) and differential scanning calorimetry results (Graham et al., 1985).

The temperature dependence of the first spectral moment of DMPA/Ca^{2+} spectra is also shown in Figure 2. At low temperatures, M_1 values of spectra of DMPA/Ca^{2+} complexes are higher than those of pure DMPA dispersions, indicating that the movements of the lipid acyl chains are highly restricted, as mentioned above. When the temperature of the DMPA/Ca^{2+} system is increased, M_1 decreases slightly up to 65 °C and more rapidly above this temperature (Figure 2). No cooperative phase transition is observed, in agreement with previous data obtained by differential scanning calorimetry (Van Dijck et al., 1978; Liao & Prestegard, 1981; Graham et al., 1985) and Raman spectroscopy (Kouaouci et al., 1985), and the M_1 values are always higher for the DMPA/Ca^{2+} system than for pure DMPA dispersions. The M_1 decrease above 65 °C can be accounted for by the appearance of the "isotropic" NMR line. Indeed, the measured M_1 value is the

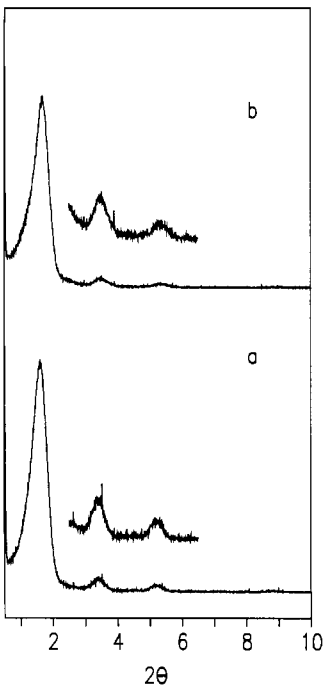

FIGURE 3: Small-angle X-ray diffraction patterns of DMPA/Ca^{2+} complexes at (a) 25 and (b) 85 °C.

weighted average of the first moment of both the broad spectrum and the narrow line. It is important to mention here that the temperature dependence of the NMR spectrum of DMPA/Ca^{2+} complexes is fully reversible, so that none of the spectral changes are due to thermal degradation of the lipid.

The type of structure adopted by DMPA/Ca^{2+} complexes at low and high temperatures was also investigated by small-angle X-ray diffraction. The diffraction pattern obtained at 25 °C (Figure 3) exhibits regularly spaced peaks corresponding to diffraction orders characteristic of the lamellar phase. The lamellar repeat distance calculated from these peaks is 52.0 ± 0.5 Å which is in good agreement with the data of Liao and Prestegard (1981). This short spacing in the presence of calcium suggests a high degree of dehydration of the bilayers.

At high temperature (85 °C), the X-ray diffraction pattern for the DMPA/Ca^{2+} complexes (Figure 3) is almost identical with that at low temperature, the lamellar repeat distance being 51.0 ± 0.5 Å. Therefore, the DMPA/Ca^{2+} system still exists as bilayers at high temperature. Furthermore, ^{31}P NMR spectra of DMPA/Ca^{2+} complexes below and above the normal phase transition temperature of DMPA (results not shown) exhibit the typical band shape of phospholipid systems in a lamellar phase.

In order to gain more information on the conformation of the acyl chains of DMPA in the presence and absence of calcium, Raman spectra were also recorded (Figure 4). The C–C stretching mode region of phospholipid dispersions exhibits three well-defined bands near 1070, 1090, and 1130 cm^{-1}. The bands at 1070 and 1130 cm^{-1} are characteristic of the almost all-trans conformation of the highly ordered acyl chains found in the gel phase of saturated phospholipids while the band at 1090 cm^{-1} is representative of the gauche conformers of the C–C skeleton (Gaber & Peticolas, 1977; Yellin & Levin, 1977).

As seen in Figure 4 (left), there is a marked increase of the 1090 cm^{-1} feature with respect to the 1070 and 1130 cm^{-1} components at the gel-to-fluid transition of DMPA, reflecting an increase of the number of gauche conformers of the C–C skeleton. On the other hand, in the presence of calcium, no

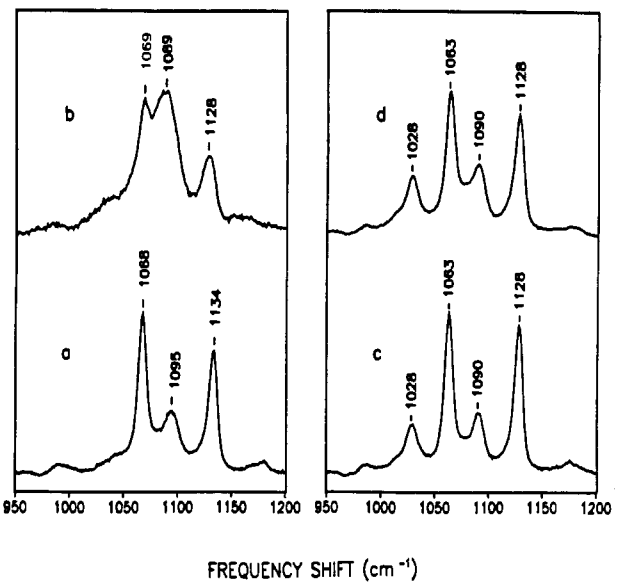

FIGURE 4: Raman spectra in the C–C stretching mode region of dispersions of DMPA at (a) 20 and (b) 60 °C and of DMPA/Ca^{2+} complexes at (c) 20 and (d) 60 °C.

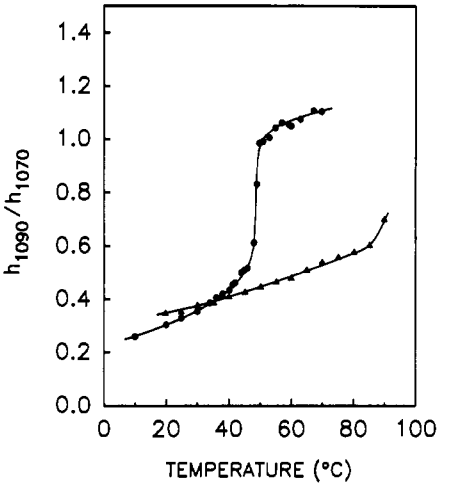

FIGURE 5: Temperature profiles derived from the Raman h_{1090}/h_{1070} peak height intensity ratio of DMPA in the absence (●) and presence (▲) of calcium ions.

significant change of spectrum occurs with temperature, the DMPA/Ca^{2+} spectrum being almost identical with that of the pure lipid at low temperature. The peak at 1028 cm^{-1} originates from a phosphate group vibration and is discussed below.

The h_{1090}/h_{1070} peak height intensity ratio is particularly useful to follow the evolution of the intramolecular order of lipid acyl chains (Gaber & Peticolas, 1977; Yellin & Levin, 1977). Figure 5 shows that pure DMPA undergoes its gel-to-fluid phase transition at 47 °C in agreement with the above NMR results and that the addition of calcium ions promotes a very important ordering effect at high temperature. In the presence of calcium, the transition is totally abolished, and the h_{1090}/h_{1070} intensity ratio increases only slightly from 20 to 90 °C. By comparison with the temperature profile of the pure lipid, it is clear that the lipid acyl chains of the DMPA/Ca^{2+} complexes remain predominantly in the all-trans conformation over the whole range of temperature investigated even though some gauche conformations are introduced along the chains.

The description of a model for the interaction between calcium and DMPA requires knowledge of the stoichiometry

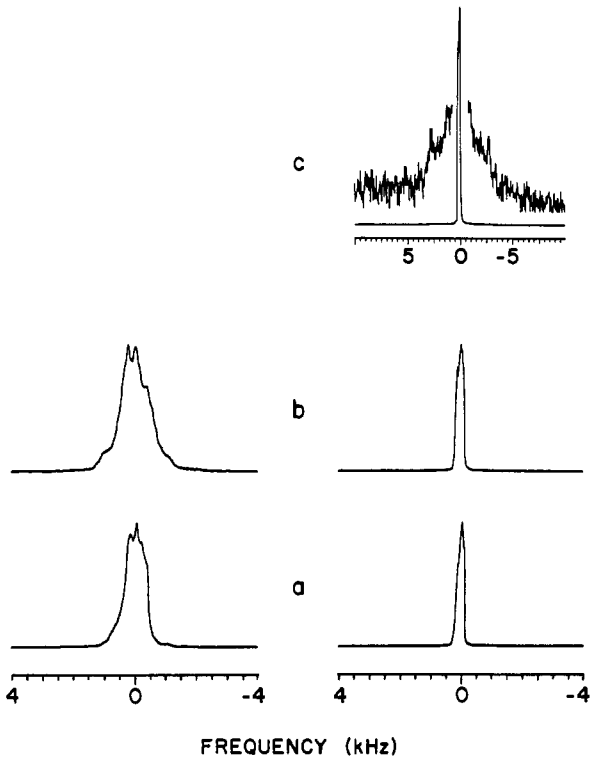

FIGURE 6: ^2H NMR spectra of ^2H_2O in DMPA dispersions (25 water molecules per lipid) in the absence (left) and presence (right) of calcium (the calcium to DMPA molar ratio was 1:2) at (a) 25, (b) 55, and (c) 25 °C, the upper trace spectrum corresponding to an increase of the vertical scale by a factor of 32.

of the system. Previous results obtained by differential scanning calorimetry (Liao & Prestegard, 1981) and by atomic absorption spectroscopy (Bicknell-Brown et al., 1986) on the interaction of calcium ions with DMPA or DPPA, respectively, show that at neutral pH each phosphatidic acid molecule binds one Ca^{2+} ion. On the other hand, the potentiometric results of Boughriet et al. (1988) indicate that at pH 4 the binding stoichiometry is two lipid molecules per calcium ion in the case of DMPA.

In order to determine the binding stoichiometry under the experimental conditions used in the current study, Raman spectra in the C–H stretching mode region of DMPA/Ca^{2+} complexes with different calcium to lipid mixing molar ratios were recorded at approximately 10 °C above the gel-to-fluid phase transition temperature of pure DMPA. It has already been shown that either the h_{2880}/h_{2850} (Laroche et al., 1988) or the h_{2935}/h_{2880} (Bicknell-Brown et al., 1986) peak height intensity ratios provide excellent probes for the determination of the binding stoichiometry of ions or polyions with phosphatidic acid. The results obtained from such a Raman titration curve for the DMPA/Ca^{2+} system (data not shown) demonstrate clearly that the h_{2880}/h_{2850} peak height intensity ratio reaches a "plateau" for a calcium to DMPA molar ratio of 1. Therefore, these results are consistent with a 1:1 binding stoichiometry at neutral pH, in good agreement with the results of Liao and Prestegard (1981) and Bicknell-Brown et al. (1986).

Structure and Dynamics of the Interfacial Region. Since the addition of calcium to DMPA bilayers leads to a massive precipitation of the complexes and to an important reduction of the lamellar repeat distance as measured by X-ray diffraction, NMR spectroscopy of ^2H_2O has been used to characterize the state of water at the interface. Figure 6 displays the ^2H NMR spectra of pure protoated DMPA dispersed in ^2H_2O at a molar ratio of 25 water molecules per lipid

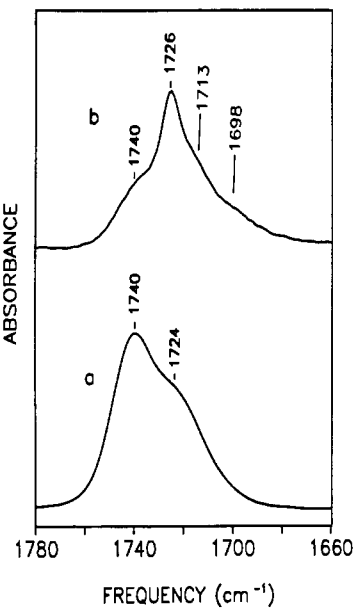

FIGURE 7: Infrared spectra in the carbonyl stretching mode region of DMPA at 20 °C in (a) the absence and (b) the presence of calcium ions.

in the presence (right) and absence (left) of calcium. In the absence of calcium, spectra are the superimposition of at least two deuterium powder patterns at temperatures below and above T_c. It is worth mentioning that the slight distortion of the spectra is due to the use of sample tubes that are much smaller than the detection coil. Nevertheless, the observed spectral features indicate that water is motionally restricted and distributed in several sites in which the molecules have different dynamic properties. Therefore, most water molecules undergo anisotropic reorientation except for the few free water molecules giving rise to the small isotropic component detected in the middle of each spectrum.

Spectra in the presence of calcium (right) display the characteristics of water reorienting freely in solution. Therefore, this is straightforward evidence that almost all water molecules which were hydrating the DMPA polar head group are now free from interacting with the lipid interface. This occurs at both low and high temperatures. However, Figure 6c obtained after increasing the vertical scale of the spectrum in the presence of calcium at 25 °C clearly reveals that there are still some water molecules (ca. 5–10%) that are very motionally restricted, i.e., bound to the interface. A similar result is also observed at 55 °C (expansion not shown).

The infrared spectra of DMPA reveal that the carbonyl and phosphate group regions are also affected by the presence of calcium. Figure 7 shows the carbonyl stretching mode region of the infrared spectra of DMPA in the absence and in the presence of calcium ions. For the pure lipid, two spectral features are observed in this region near 1740 and 1724 cm^{-1}. Results using selectively labeled $^{13}C{=}O$ phospholipids and the model ester compound ethyl acetate in different solvents (Blume et al., 1988; our results, unpublished data) show that a splitting of the carbonyl band occurs only when hydrogen bonding is possible between the ester group and the solvent. Even though the assignment of these two bands is still a matter of controversy (Green et al., 1987), it is likely that the low-frequency contribution is due to carbonyl group that is hydrogen bonded to water while the high-frequency contribution is associated with free carbonyls, independently of the chain bearing the C=O groups.

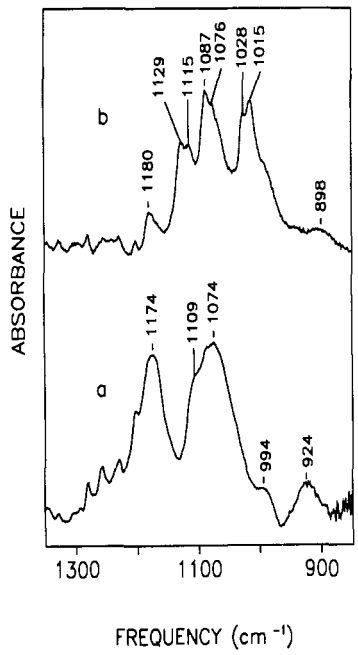

FIGURE 8: Infrared spectra in the phosphate stretching mode region of DMPA at 25 °C in (a) the absence and (b) the presence of calcium ions.

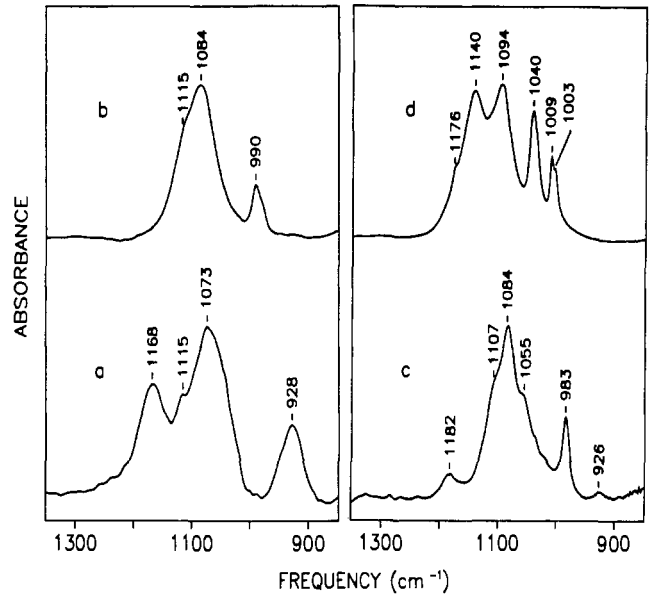

FIGURE 9: Infrared spectra at 25 °C of the phosphate stretching mode region of DHPA at (a) pH 6.0 and (b) pH 10.0 and of MMP in (c) the absence and (d) the presence of calcium ions.

As seen in Figure 7, the infrared spectrum of the pure DMPA dispersions shows that the intensity of the 1740 cm^{-1} feature is stronger than the 1724 cm^{-1} component, thus indicating that the fraction of carbonyl groups that are hydrogen bonded to water for this lipid is slightly smaller than the fraction of free carbonyls. The addition of calcium to the lipid bilayer promotes a drastic reorganization of the lipid interfacial region since the absorbance of the 1726 cm^{-1} band becomes much higher than that of the 1740 cm^{-1} component. Although these results do not rule out the possibility of direct interaction between the lipid carbonyl groups and calcium, the results suggest that more carbonyl groups are hydrogen bonded to water in the presence of calcium. At least two additional components are also present in the C=O stretching mode region of the spectrum of DMPA/Ca^{2+} complexes at approximately 1713 and 1698 cm^{-1}. The infrared data of Patel et al. (1985) on the solvation of esters and carbonates in various solvents indicate that these bands could be assigned to C=O groups hydrogen bonded to two and three water molecules, respectively. The strong perturbation of the band shape of the spectral feature due to the bending mode of water in the infrared spectrum of the DMPA/Ca^{2+} system (result not shown) further supports the existence of strongly bound water molecules.

Figure 8 displays the phosphate stretching mode region of the infrared spectra at 25 °C of a pure DMPA dispersion at pH 6.5 and of DMPA/Ca^{2+} complexes. Although this spectral region is not fully understood because of the presence of several overlapping bands, the features observed at 1174 and 1074 cm^{-1} in the infrared spectrum of pure DMPA can be assigned unambiguously to the PO_2^- antisymmetric and symmetric stretching modes, respectively, by comparison with spectra of organophosphorus compounds (Thomas & Chittenden, 1970) and other phospholipids (Casal et al., 1987a,b,c). The weaker bands superimposed with the PO_2^- antisymmetric stretching component are due to the acyl chains' methylene wagging progression (Casal & Mantsch, 1984) and demonstrate that the acyl chains are mainly in the all-trans conformation as observed from the Raman spectra in the C–C stretching mode region. Finally, this spectral region also exhibits features characteristic of the C–O–(P), P–O–(C), and P–O–(H) stretching modes. According to the analysis of Thomas and Chittenden (1963), the bands at 1109 and 994 cm^{-1}, that overlapped with the band due to the PO_2^- symmetric stretching mode in the spectrum of DMPA, could be assigned to the C–O–(P) and the P–O–(C) stretching vibrations, respectively, while the 924 cm^{-1} band is most likely due to the P–O–(H) stretching mode.

The infrared spectrum in the phosphate stretching mode region of DMPA/Ca^{2+} complexes (Figure 8b) exhibits a very complex pattern. Three doublets are observed at 1115 and 1129, 1076 and 1087, and 1015 and 1028 cm^{-1}, in addition to two weak features at 1180 and 898 cm^{-1}. This complex spectrum is not easily explained on the basis of the assignments given above only, and its elucidation has required further studies on some model compounds. Since the phosphate stretching mode region of the infrared spectra of phospholipids should be sensitive to the number of charges borne by the phosphate group, and also because the interaction of calcium ions with DMPA may also influence the charge density of this group, the infrared spectra of DHPA at pH 6 and at pH 10 were recorded. This ether lipid was chosen instead of DMPA in order to prevent hydrolysis of the ester group at high pH.

At pH 6, phosphatidic acid bears only one negative charge while at pH 10 the phosphate group is doubly ionized (Träuble & Eibl, 1974; Galla & Sackmann, 1975). Figure 9a displays the phosphate stretching mode region of the infrared spectrum of DHPA at pH 6. This spectrum exhibits essentially the same features as those observed for the pure DMPA dispersion (Figure 8a), the antisymmetric and symmetric PO_2^- stretching modes occurring at 1168 and 1073 cm^{-1}, respectively. At pH 10 (Figure 9b), the spectrum of DHPA is quite different in this region because the additional charge of the head group leads to a change of symmetry of the phosphate group which is then isomorphic with the C_{3v} point group. In this case, the bands due to the symmetric and asymmetric PO_3^{2-} stretching modes arise at 990 and 1084 cm^{-1}, respectively, in good agreement with the results of Thomas and Chittenden (1970) on organophosphorus compounds. As mentioned above, the feature observed at 1115 cm^{-1} is assigned to the C–O–(P) stretching vibration. The assignment of the 990 cm^{-1} feature

to the PO_3^{2-} symmetric stretching vibration in the infrared spectrum of DHPA at pH 10 is further supported by the fact that this vibrational mode is also Raman-active (results not shown) as expected for the C_{3v} point group. The disappearance of the 928 cm^{-1} feature at pH 10 confirms the assignment of this band to the P–O–(H) stretching mode in the spectrum of DHPA at pH 6 and of DMPA at pH 6.5.

In order to determine whether calcium ions bind to one or to both charges of DMPA, the infrared spectra of dicyclohexylammonium monomethyl phosphate (MMP) in the absence and in the presence of calcium ions were recorded. The simplicity of this molecule allows the unambiguous assignment of the phosphate vibrations since the infrared absorption observed at 850–1350 cm^{-1} can arise only from the phosphate group. Moreover, this salt bears two negative charges on the phosphate group. Figure 9c shows the phosphate stretching mode region of the infrared spectrum of MMP in the absence of calcium. This spectrum is quite similar to that of DHPA at pH 10, indicating that the phosphate group of this salt effectively bears two negative charges. However, the two weak features at 1182 and 926 cm^{-1} indicate that some molecules are singly charged.

When MMP is dissolved in a solution containing $CaCl_2$, the binding of calcium ions on the phosphate group leads to the formation of a white precipitate. The infrared spectrum of the pelleted sample presented in Figure 9d is quite similar to that of the DMPA/Ca^{2+} system (Figure 8b), suggesting that DMPA bears two charges when it interacts with calcium ions. Moreover, this spectrum is very similar to those of dicalcium phosphate dihydrate (Berry & Baddiel, 1967; Petrov et al., 1967) and anhydrous dicalcium phosphate (Petrov et al., 1967). It has been suggested that the binding of Ca^{2+} to PO_4^{3-} ions in these salts lowers the symmetry of the phosphate group from C_{3v}, where the PO_3^{2-} asymmetric stretching vibration is doubly degenerate, to a very low symmetry point group for which the degeneracy is removed and all the modes are infrared-active. Therefore, on the basis of the assignments of Berry and Baddiel (1967) and of Petrov et al. (1967), one can conclude that the infrared bands observed as doublets at 1115 and 1129, 1076 and 1087 cm^{-1} for the DMPA/Ca^{2+} complexes and at 1140 and 1094 cm^{-1} for the MMP/Ca^{2+} complexes are due to the asymmetric stretching vibrations of the PO_3^{2-} group of symmetry lower than C_{3v}, while the doublet observed at 1015 and 1028 cm^{-1} for DMPA/Ca^{2+} complexes and the 1040 cm^{-1} band for the MMP/Ca^{2+} system arise from the PO_3^{2-} symmetric stretching mode. The assignment of the PO_3^{2-} symmetric stretching vibration is further confirmed by the appearance in the Raman spectra of these complexes of bands at 1028 cm^{-1} for DMPA/Ca^{2+} complexes (Figure 4) and at 1035 cm^{-1} for the MMP/Ca^{2+} system (results not shown), since as mentioned above, the PO_3^{2-} symmetric stretch is also Raman-active. The absence of this feature in the Raman spectrum of pure DMPA dispersions at pH 6.5 clearly demonstrates that the phosphate group of this lipid bears only one charge at physiological pH. It is also interesting to note that the three bands due to the PO_3^{2-} stretching vibrations are split in two components in the infrared spectra of DMPA/Ca^{2+} complexes. A similar splitting has been observed by Petrov et al. (1967) in the infrared spectra of dicalcium phosphate dihydrate and was assigned to a correlation field splitting.

Finally, it seems that the replacement of the proton of the P–OH group by the calcium ions is not complete since infrared bands due to the antisymmetric PO_2^- and P–O–(H) stretching modes are still present at 1180 and 898 cm^{-1}, respectively, for DMPA/Ca^{2+} complexes and at 1176 cm^{-1} and 1003 and 1009 cm^{-1} for MMP/Ca^{2+} complexes.

Discussion

The use of several powerful techniques such as NMR, Raman, and infrared spectroscopies and X-ray diffraction provides new insight, at the molecular level, about the structure of complexes formed between calcium ions and the anionic phospholipid DMPA. The results obtained clearly indicate that calcium changes both the acyl chain packing and the structure of the interfacial region. These two aspects will be discussed successively.

The ^2H NMR results on pure DMPA dispersions show that this lipid undergoes a typical gel-to-fluid phase transition around 47 °C which is in good agreement with previously published results on this lipid (Mushayakarara & Levin, 1984; Graham et al., 1985; Laroche et al., 1988, 1990). When calcium ions are added to the phospholipid dispersion, the shape of the ^2H NMR spectra at low temperature demonstrates clearly that the DMPA acyl chains become more ordered since the width of the spectra of the DMPA/Ca^{2+} complexes is broader than that of pure DMPA. This is also reflected on the first moment of the ^2H NMR spectra at low temperature which is higher for DMPA/Ca^{2+} complexes than for the pure lipid. Such an ordering effect of calcium ions on the acyl chains of anionic phospholipids has also been reported by several workers (Susi, 1981; Hark & Ho, 1980; Dluhy et al., 1983; Kouaouci et al., 1985; Graham et al., 1985) but had never been investigated by ^2H NMR spectroscopy.

The temperature dependence of the results obtained by ^2H NMR and Raman spectroscopies shows that DMPA/Ca^{2+} complexes do not undergo any cooperative phase transition in the temperature range investigated, which is in good agreement with previous differential scanning calorimetry investigations (Van Dijck et al., 1978; Liao & Prestegard, 1981; Graham et al., 1985). However, the first moment of the ^2H NMR spectrum decreases significantly above 65 °C (Figure 2) while the Raman results (Figure 5) show that there is only a slight increase in the number of gauche bonds at high temperature. The X-ray results on complexes of DMPA with calcium ions provide conclusive evidence that the bilayer structure is preserved at all temperatures investigated since the diffraction pattern characteristic of the lamellar phase is always observed and does not change with temperature. This behavior is different from that of dioleoylphosphatidic acid (DOPA) which has been shown to adopt an inverted hexagonal structure in the presence of calcium (Verkleij et al., 1982). This can easily be rationalized by using the concept of "equivalent geometry" proposed by Israellachvili et al. (1977), Cullis and de Kruijff (1979), and Siegle (1986a,b). The longer and unsaturated chain of DOPA must statistically occupy a too large volume to fit in a lamellar phase where phosphate groups are tightly packed in the presence of Ca^{2+}.

The results presented above lead to the following model for the description of the thermotropic behavior of DMPA/Ca^{2+} complexes. Calcium ions interact strongly with the polar head group of DMPA, and this electrostatic interaction leads to a rigidification of the lipid acyl chains. This effect has also been detected, but to a much lesser extent, when basic or amphipathic peptides interact with anionic phospholipids (Laroche et al., 1990; Roux et al., 1989; Dufourc et al., 1986) and has been attributed to the capping of the polar head group of these lipids. All techniques are consistent with this description except for ^2H NMR at high temperatures which indicates that above 65 °C the quadrupolar interaction is significantly reduced. This effect cannot be attributed to the appearance of a new phase of higher symmetry or to an increase in gauche con-

formers as evidenced by X-ray (Figure 3) and Raman (Figure 5) results, respectively. Since ^2H NMR spectra are sensitive to motions in the microsecond time scale or faster, it is likely that the spectral narrowing detected at high temperatures results from the activation of very slow motions. The appearance of an "isotropic" line in ^2H NMR spectra requires motion with spherical symmetry. Although our results do not provide complete characterization of such a motion, one may postulate isotropic reorientations of entire lamellar domains are activated at high temperature.

This study also shed more light on the behavior of the water molecules in the DMPA/Ca^{2+} system. The relatively small interlamellar repeat distance determined by X-ray diffraction reveals that adjacent bilayers are much closer in the presence of calcium ions, suggesting that most water molecules are removed from the interbilayer space. A similar decrease of the lamellar repeat distance has also been observed for complexes of calcium with DMPA (Liao & Prestegard, 1981) or egg PG (Murthy et al., 1984). This is further inferred by the ^2H_2O NMR results which demonstrate that the DMPA molecules are highly dehydrated in the presence of calcium ions. In addition, the NMR results show that some water molecules are strongly immobilized in the DMPA/Ca^{2+} complexes. The infrared data in the carbonyl stretching mode region suggest that these water molecules are hydrogen bonded to the carbonyl groups of the lipid ester group.

The analysis of the phosphate stretching mode region of the infrared spectra of DMPA/Ca^{2+} complexes also gives valuable information about the structural organization of the phospholipid molecules in this system. Comparison of the infrared spectrum of DMPA/Ca^{2+} (Figure 8) complexes with those of model compounds (Figure 9) unambiguously demonstrates that calcium ions remove almost all the protons borne by the DMPA molecules at pH 6.5, as suggested in previous studies (Abramson et al., 1965; Hauser & Dawson, 1967; Barton, 1968), and, therefore, bind to the three oxygen atoms on which the two negative charges are delocalized. The similarities between the phosphate stretching mode region of the infrared spectrum of DMPA/Ca^{2+} complexes with those of dicalcium phosphate dihydrate (Berry & Baddiel, 1967; Petrov et al., 1967) and anhydrous dicalcium phosphate (Petrov et al., 1967) lead to the conclusion that calcium ions bind to the phosphate group of the DMPA molecules to form a similar network as observed in these dicalcium phosphate salts.

The crystal structure of these salts determined by Maclennan and Beevers (1955) and Beevers (1958) reveals that the oxygen atoms are shared by the calcium ions on each side to give a continuous chain of Ca and P atoms linked by the oxygen atoms. In addition to these four Ca bonds to oxygen atoms within the chain, each Ca atom is bonded to two oxygens in neighboring chains.

It is clear that a similar network cannot be found in the DMPA/Ca^{2+} complexes since one oxygen atom of the phosphate group is esterified to the glycerol backbone of the lipid molecule. However, the correspondence of the features in the infrared spectra of dicalcium phosphate salts (Berry & Baddiel, 1967; Petrov et al., 1967) and DMPA/Ca^{2+} complexes strongly suggests that calcium ions should bind to the three oxygen atoms of the phosphate group of DMPA molecules. However, the infrared results indicate that the Ca–O bonds are stronger in the inorganic salts since the frequency of the PO_3^{2-} symmetric stretching mode is significantly lower in the spectra of these salts than in that of the DMPA/Ca^{2+} system.

Several authors have already proposed that calcium ions act as a bridge between two adjacent bilayers (Papahadjopoulos, 1968; Lee, 1977; Liao & Prestegard, 1981). Papahadjopoulos (1968) has hypothesized that the interaction between calcium ions and phosphatidic acid involves coordination bonds between each Ca^{2+} ion and four lipid molecules, resulting in a linear polymeric arrangement in which the two negative charges borne by the PA head group in the presence of calcium are localized on two of the three oxygen atoms of the phosphate group. This model, which accounts for the 1:1 stoichiometry, is not compatible with the infrared results that clearly demonstrate that both negative charges are delocalized on the three oxygen atoms.

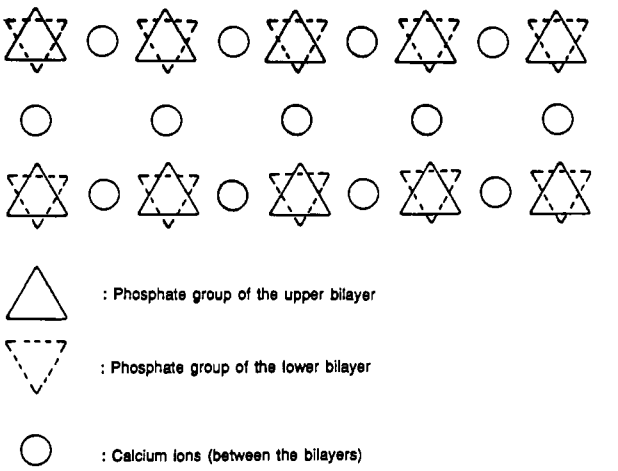

FIGURE 10: Proposed model for phosphate arrangement of the lamellar structure of DMPA in the presence of calcium. Each triangle represents the three oxygen atoms of a phosphate group.

A two-dimensional network as presented in Figure 10 can better account for all data presented in this paper. In this model, each calcium ion is also coordinated by four lipid molecules as proposed by Papahadjopoulos (1968). However, the PA molecules of adjacent linear arrangements are also bound by calcium ions, resulting in a two-dimensional network where the lipid molecules of adjacent bilayers are separated by a plane of calcium ions.

It is quite interesting to compare our small-angle X-ray results with the crystal structure of DMPA obtained by Harlos et al. (1984). The lamellar repeat distance of a fully dehydrated bilayer of DMPA is 44 Å when the lipid molecules are tilted with a 24° angle. Since it has been shown by Liao and Prestegard (1981) that the neutralization of the negative charges of DMPA by calcium ions should abolish the tilt angle of the lipid molecules because of the decrease of the electrostatic repulsions between the phosphate groups, simple calculations show that the bilayer thickness should increase by 4 Å if the acyl chains are perpendicular to the bilayer surface. On the other hand, a Ca–O bond length of approximately 2.5 Å as observed for dicalcium phosphate salts (Maclennan & Beevers, 1955; Beevers, 1958) implies that the calcium atom is located at approximately 1.8 Å from the bilayer surface. If twice this distance is added to the bilayer thickness of 48 Å for untilted lipid molecules, a repeat distance of 51.6 Å is calculated, a very good agreement with the value of 52.0 ± 0.5 Å found in this work. Therefore, the X-ray results presented here in addition to the striking similarities in the infrared spectra of dicalcium phosphate salts and DMPA/Ca^{2+} complexes strongly suggest that calcium ions form bridges between phosphate groups of adjacent bilayers.

However, it seems evident that several defects could be present in such a structure. In fact, the phosphate stretching mode region of the infrared spectrum of DMPA/Ca^{2+} complexes clearly demonstrates that calcium ions do not remove

all protons of the DMPA head group, thus leading to breaks in this network. Furthermore, even if the experimental data suggest that calcium ions bridge two neighboring bilayers, the possibility that Ca^{2+} ions also bridge two DMPA molecules in the same bilayer cannot be ruled out. Nevertheless, the occurrence of bridges between adjacent bilayers for at least a fraction of the lipid molecules would probably favor the formation of the cylindrical structure observed for several anionic phospholipid/calcium systems.

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Mass Spectrometric Identification of the Amino Donor and Acceptor Sites in a Transglutaminase Protein Substrate Secreted from Rat Seminal Vesicles†

Raffaele Porta,*,‡ Carla Esposito,§ Salvatore Metafora,∥ Antonio Malorni,⊥,# Pietro Pucci,⊥,▽ Rosa Siciliano,⊥,# and Gennaro Marino⊥,◇

Istituto di Chimica Biologica, Facoltà di Medicina e Chirurgia, Università di Palermo, Palermo, Italy, Dipartimento di Biochimica e Biofisica, Università di Napoli, Napoli, Italy, Istituto Internazionale di Genetica e Biofisica, CNR, Napoli, Italy, Servizio di Spettrometria di Massa, CNR, Università di Napoli, Napoli, Italy, Istituto di Chimica delle Molecole di Interesse Biologico, CNR, Arco Felice, Napoli, Italy, Dipartimento di Chimica, Università della Basilicata, Potenza, Italy, and Dipartimento di Chimica Organica e Biologica, Università di Napoli, Napoli, Italy

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ABSTRACT: Four different transglutaminase-modified forms of a protein secreted by the rat seminal vesicles (SV-IV) were synthesized in vitro and characterized. FAB maps of both the native protein and its derivatives, produced by the purified guinea pig liver enzyme in the presence or absence of the polyamine spermidine, were obtained by mass spectrometric analysis after proteolytic digestions. Two differently derivatized SV-IV molecular forms, both possessing only one glutamine residue out of two (Gln-86) cross-linked to endogenous lysine residues, were produced when spermidine was omitted from the reaction mixture: (i) an insoluble homopolymer in which Lys-2, -4, -59, -78, -79, and -80 were involved in the linkage; (ii) a soluble form of the protein with an intramolecular ε-(γ-glutamyl)lysine isopeptide bond between Gln-86 and Lys-59. Two species of SV-IV–spermidine adducts were obtained when the protein was treated with transglutaminase in the presence of high concentrations of the polyamine. The first one was characterized by one spermidine molecule covalently bound to Gln-86 and the second one by two spermidine molecules respectively bound to Gln-9 and Gln-86.

Rat seminal vesicle (SV)[1] epithelium is an androgen-dependent tissue that synthesizes five major secretory proteins designated SV-I through SV-V depending on their migration in an SDS–PAGE system (Higgins et al., 1976; Ostrowski et al., 1979; Wagner & Kistler, 1987). Among these, SV-IV (M_r = 9.758) is the most extensively studied protein; the sequence of its 90 amino acids and the general features of the gene coding for it have been determined (Pan et al., 1980; Pan & Li, 1982; Mansson et al., 1981; Harris et al., 1983; Kandala et al., 1983, 1985). As for the biological properties of the protein, SV-IV was recently found to possess immunosuppressive and anti-inflammatory activities (Metafora et al., 1989a,b; Galdiero et al., 1989). Moreover, it was suggested that SV-IV might be one of the clotting proteins that serve as substrates for transglutaminase (TGase, EC 2.3.2.13) secreted by the rat anterior prostate in the formation of the

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* Address correspondence to this author at Dipartimento di Biochimica e Biofisica, Via Constantinopoli, 16 I-80138 Napoli, Italy.
‡ Università di Palermo.
§ Dipartimento di Biochimica e Biofisica, Università di Napoli.
∥ Instituto Internazionale di Genetica e Biofisica, CNR.
⊥ Servizio di Spettrometria di Massa, CNR, Università di Napoli.
CNR, Arco Felize.
▽ Università della Basilicata.
◇ Dipartimento di Chimica Organica e Biologica, Università di Napoli.

[1] Abbreviations: SV, seminal vesicle; TGase, transglutaminase; FABMS, fast atom bombardment mass spectrometry; Spd, spermidine; TFA, trifluoroacetic acid.

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