Low pH Induces an Interdigitated Gel to Bilayer Gel Phase Transition in Dihexadecylphosphatidylcholine Membrane

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ABSTRACT We have investigated the influence of pH on the structures and phase behaviors of multilamellar vesicles of the ether-linked dihexadecylphosphatidylcholine (DHPC-MLV). This phospholipid is known to be in the interdigitated gel ($L_{\beta}I$) phase in excess water at 20°C at neutral pH. The results of X-ray diffraction experiments indicate that a phase transition from $L_{\beta}I$ phase to the bilayer gel phase occurred in DHPC-MLV in 0.5 M KCl around pH 3.9 with a decrease in pH, and that at low pH values, less than pH 2.2, DHPC-MLVs were in $L_{\beta'}$ phase. The results of fluorescence and light scattering method indicate that the gel to liquid-crystalline phase transition temperature (T_m) of DHPC-MLV increased with a decrease in pH. On the basis of a thermodynamic analysis, we conclude that the main mechanism of the low-pH induced $L_{\beta}I$ to bilayer gel phase transition in DHPC-MLV and the increase in its T_m is connected with the decrease in the repulsive interaction between the headgroups of these phospholipids. As pH decreases, the phosphate groups of the headgroups begin to be protonated, and as a result, the apparent positive surface charges appear. However, surface dipoles decrease and the interaction free energy of the hydrophilic segments with water increases. The latter effect dominates the pure electrostatic repulsion between the charged headgroups, and thereby, the total repulsive interaction in the interface decreases.

INTRODUCTION

It is well known that diacylphosphatidylcholine (PC) such as dipalmitoylphosphatidylcholine (DPPC) and dialkylphosphatidylcholine such as DHPC can form the $L_{\beta}I$ phase (Simon and McIntosh, 1984; Kim et al., 1987; Laggner et al., 1987; Slater and Huang, 1988; Yamazaki et al., 1994; Huang and McIntosh, 1997). Especially, the formation of $L_{\beta}I$ phase of diacylphosphatidylcholine in the presence of ethanol and other short-chain alcohols has been vigorously investigated (Rowe and Campion, 1994; Vierl et al., 1994; Löbbecke and Cevc, 1995; Adachi et al., 1995). Recently, we have shown that water-soluble organic solvents such as acetone, acetonitrile, propionaldehyde, and tetrahydrofurane also induce $L_{\beta}I$ phase in DPPC-MLV (Kinoshita and Yamazaki, 1996). These results demonstrated that a specific interaction of alcohols with phospholipid membranes is not important in the formation of the $L_{\beta}I$ phase. Factors that play important roles in the formation of the $L_{\beta}I$ phase and the $L_{\beta'}$ to $L_{\beta}I$ phase transition include interactions between the interfaces of these membranes and solvents as well as the interaction between the headgroups of phospholipids (Simon and McIntosh, 1984; Rowe and Campion, 1994; Kinoshita and Yamazaki, 1996).

Recently, dialkylphospholipids and alkyl-acyl-phospholipids that contain ether-linkages have attracted much attention as a platelet-activating factor and as an antitumor activity (Snyder et al., 1985; Lohmeyer and Bittman, 1994), and also as major lipids of archaebacterial membranes (Bloom and Mouritsen, 1995). An ether-linked dialkylphospholipid, DHPC, has a very similar molecular structure to that of an ester-linked diacylphospholipid, DPPC, and the small difference in their molecular structures is that DPPC has additionally two C═O groups and DHPC does not. These PC membranes have different phase behaviors, and especially, DPPC-MLV is in the bilayer gel phase in excess water at 20°C at neutral pH, whereas DHPC-MLV under the same conditions is in $L_{\beta}I$ phase (Kim et al., 1987; Laggner et al., 1987). The large repulsion between the headgroups has been considered as a main reason for the formation of $L_{\beta}I$ phase in DHPC-MLV at neutral pH (Hatanaka et al., 1997). Despite the intensive investigation of the mechanism of the formation of $L_{\beta}I$ phase, the effect of interaction between the headgroups on the stability of $L_{\beta}I$ phase is still not well understood.

The pH-titration is a fruitful method for investigation of the effect of the electrostatic interaction (or repulsion) between surface charges of phospholipid membranes and proteins. This method gives the opportunity to vary the surface charge density without change of the chemical structure and the size of the surface segments. It has helped to elucidate our understanding of phases and colloid behaviors of the charged phospholipid membranes such as phosphatidic

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Abbreviations used: DSC, differential scanning calorimetry; DHPC, 1,2-dihexadecyl-*sn*-glycero-3-phosphatidylcholine; L_{α} phase, liquid-crystalline phase; $L_{\beta'}$ phase, bilayer gel phase with tilted hydrocarbon chains; $L_{\beta}I$ phase, interdigitated gel phase; MLV, multilamellar vesicles; $P_{\beta'}$ phase, ripple phase; SAXS, small-angle X-ray scattering; WAXS, wide-angle X-ray scattering; PC, phosphatidylcholine.

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acid, phosphatidylserine, and phosphatidylglycerol. Particularly, the change of gel to liquid-crystalline phase transition temperatures of these charged membranes has been investigated vigorously, and explained reasonably by a theory based on the Gouy–Chapmann diffuse-double layer theory (Träuble et al., 1976, Jähnig et al., 1979; Watts et al., 1981; Cevc et al., 1981). However, in contrast, the effect of pH on the phase behavior of PC membranes, which have zero net charges at neutral pH, is not well understood yet.

The main aim of the present study is to investigate the influence of pH on the phase stability of DHPC-MLV. We have found that, in DHPC-MLV, a phase transition from $L_\beta I$ to bilayer gel phase occurred around pH 3.9, and that, at low pH, DHPC-MLVs were in the bilayer gel phase. We have also demonstrated that the gel to liquid-crystalline phase transition temperature (chain-melting temperature) T_m increased with a decrease in pH. On the basis of a thermodynamic analysis, we conclude that the main mechanism of this phase transition is connected with the decrease in the repulsive interaction between the headgroups of DHPC molecules with a decrease in pH. As pH decreases, phosphate groups of DHPC molecules begin to be protonated. As a result, the apparent positive surface charges appear. They increase the electrostatic repulsion between the headgroups, but, at the same time, the interaction free energy of the hydrophilic segments of the headgroups with water increases, which results in the decrease of the repulsion. The decrease in the repulsive interaction due to the increase in the interaction free energy dominates over the increase in the electrostatic repulsion due to the positive surface charge. Thereby, the total repulsive interaction in the polar region decreases with decreasing pH.

MATERIALS AND METHODS

Materials and sample preparation

DHPC was purchased from Fluka Chemie AG (Buchs, Switzerland). MLVs were prepared by adding appropriate amounts of various pH buffers to dry lipids (in excess water), and the suspensions were vortexed for about 30 sec around 55°C several times. For pH 6.0 $\leq$ pH $\leq$ pH 7.0, 10 mM PIPES buffer, and for pH 2.5 $<$ pH $<$ pH 6.0, 20 mM citrate buffer were used. These buffers contained various concentrations of KCl. For pH 1.2 $\leq$ pH $\leq$ pH 2.5, HCl/KCl buffers with various ionic strength (I) were used. For measurements of x-ray diffraction, pellets (~50 wt% lipid) after the centrifugation (14,000 $\times$ g, 1 h at 20°C; Tomy, MR-150, Tokyo, Japan) of the suspensions of 1 mM DHPC-MLV were used. The pH values of the suspensions were rechecked by measuring pH of their supernatants after the centrifugation. Hydrolysis of phospholipids at low pH was checked after the x-ray diffraction measurements by thin-layer chromatography using TLC plates (MERCK, Silica gel 60, Darmstadt, Germany) with the solvent system $CHCl_3/CH_3OH/H_2O$ (65:25:4, v/v). Under the conditions we used in this report, no hydrolysis was observed.

X-ray Diffraction

X-ray diffraction experiments were performed by using a Nickel-filtered Cu K_α-radiation (λ = 0.154 nm) from the rotating anode type x-ray generator (Rigaku, Rotaflex, RU-300, 50 kV $\times$ 300 mA, Tokyo, Japan). SAXS data were recorded using a linear [one-dimensional (1D)] position-sensitive proportional counter (PSPC) (Rigaku, PSPC-5) (Glatter and Kratky, 1982) with camera length of 350 mm and associated electronics (multichannel analyzer, etc., Rigaku). WAXS patterns were recorded by using a 1D PSPC with the sample-to-detector distance of 250 mm, and diffraction spacings were calibrated by using a polyethylene (Geil, 1963). In all cases, samples were sealed in a thin-walled glass capillary tube (outer diameter 1.0 mm) and mounted in a thermostatable holder whose stability was $\pm$0.2°C (Yamazaki et al., 1992).

SAXS data were processed by a standard method (McIntosh, 1980; Kinoshita et al., 1998). Integrated intensities of various diffraction peaks, $I(h)$, where h is the order number, were determined after background subtraction. Measured intensities are corrected by multiplying by the square of the order number, h^2, for a powder pattern (unoriented samples) and a correction factor, $P(h)$, due to the geometry of the 1D PSPC (Glatter and Kratky, 1982). Hence, the structure amplitude, $F(h)$, equals $\sqrt{h^2 I(h) P(h)}$. Electron density distributions, $\rho(x)$, were calculated by

$$\rho(x) \propto \sum \sqrt{h^2 I(h) P(h)}\, j(h) \cos(2\pi h x/d), \qquad (1)$$

where $j(h)$ is the phase information for each order h, and d is a spacing. For a centrosymmetric $\rho(x)$ function, $j(h)$ must be either $+1$ or -1 for each order h.

Measurement of phase transition temperatures by fluorescence spectroscopy

Phase transition temperatures of DHPC-MLV were monitored by a fluorescent probe, *N*-phenylnaphtylamine (NPN). A quantum yield of the fluorescence of NPN depends on the polarity of the solvent surrounding the NPN molecule; in a nonpolar solvent, in which dielectric constant is low, its fluorescence is enhanced (Träuble et al., 1976; Lakowicz, 1983). Träuble et al. indicated that a phase transition of phospholipid membranes from the gel to liquid-crystalline phase was accompanied by a large, abrupt increase in the fluorescence intensity of NPN. This is due to the higher partitioning of NPN into the liquid-crystalline phase and the higher quantum yield of NPN incorporated into the membrane compared with NPN in water. The phase transition temperatures were determined as temperatures at the half point of the large, abrupt increase in fluorescence intensity at the phase transitions (Träuble et al., 1976). The accuracy of the phase transition temperatures determined by this method is $\pm$0.5°C.

For fluorescence measurement, a Hitachi F3000 spectrofluorimeter (Hitachi, Tokyo, Japan) was used. The excitation wavelength was 350 nm and the emission wavelength was 430 nm. Excitation bandpass and emission bandpass were 3 nm and 1.5 nm, respectively. As a sample, DHPC-MLV dispersions in various kinds of buffers were used. Concentrations of phospholipid (Barlett, 1959) and NPN were 1.0×10^{-4} M and 1.0×10^{-6} M, respectively. Each sample (2-ml) was heated from 25°C at a rate of 0.5°C/min using an external, computer-controlled circulating water bath (NESLAB, ENDOCAL RTE-110NH).

Light scattering

For light scattering measurement, a Hitachi F3000 spectrofluorimeter was used. The wavelength and the angle of scattering were 450 nm and 90°, respectively. Concentrations of phospholipid were 1.0×10^{-4} M. Each sample (2-ml) was heated from 25°C at a rate of 0.5°C/min using an external, computer-controlled circulating water bath (NESLAB, ENDOCAL RTE-110).

Differential scanning calorimetry

Differential scanning calorimetry experiments were performed using a Rigaku DSC-8230B instrument. DHPC-MLV dispersion (1.4 wt% lipid) were heated at a rate of 2.0°C/min. Main transition temperature and pretransition temperature of DHPC-MLV were determined as the onset of

the endothermic transition extrapolated to the baseline. The details were described in our previous paper (Yamazaki et al., 1992).

RESULTS

Structural changes of DHPC-MLV induced by low pH

DHPC-MLV in excess water at 20°C is known to be in the $L_\beta I$ phase (Kim et al., 1987; Laggner et al., 1987; Hatanaka et al., 1997). To investigate the effects of pH on the structures of DHPC membrane, we have carried out the x-ray diffraction experiments such as SAXS and WAXS for DHPC-MLVs at various pH buffers (ionic strength, $I \approx 0.5$). As shown in Fig. 1, the spacing (lamellar repeat period, d) of DHPC-MLV at 20°C at neutral pH was 5.0 nm, and rapidly increased at pH 3.9 with decreasing pH. A sudden and large increase of the spacing suggests that a phase transition occurred in the DHPC-MLV. At a pH region (pH 3.5∼3.9), two kinds of the first-order diffraction peaks were observed. The first peaks of DHPC-MLVs at a pH region from 2.2 to 3.9 were broader peaks, and their spacings were a little larger than those at a low pH region, <2.2.

Generally, phosphatidylcholine (PC) membranes have no net charges at neutral pH, but at low pH (<∼2), a phosphate group of the headgroup of PC is protonated, and thereby, the PC membranes have positive charges. To confirm this, the dependence of the spacing of DHPC-MLV on KCl concentration was investigated by SAXS. Figure 2 shows that the spacing of DHPC-MLV at pH 1.5 increased with a decrease in KCl concentration.

We have determined electron density profiles of the DHPC-MLVs by using Eq. 1 in the Materials and Methods section. A set of phases, $j(h)$, at pH 7.0 is known as (−1, −1, +1) for orders $h = 1$ to 3 (Kim et al., 1987), and $j(h)$ at pH 1.5 was determined as (−1, −1, +1, −1) for orders $h = 1$ to 4, which is the same as that of $L_{\beta'}$ phase of DHPC-MLV under several conditions (Hatanaka et al., 1997; Takahashi et al., 1997). By using these phases, electron density profiles of DHPC-MLV at pH 7.0 and pH 1.5 were obtained (Fig. 3). They show that the distances between headgroup peaks across the bilayer, $d_{p\text{-}p}$, are 3.2 nm at pH 7.0, and 4.9 nm at pH 1.5. A WAXS pattern at pH 7.0 at 20°C consisted of a sharp reflection at 0.409 nm, showing that alkyl chains were packed in a hexagonal arrangement without any inclination. In contrast, at pH 1.5, it consisted of a relatively symmetrical peak centered at 0.419 nm, which is broader than that of phosphatidylethanolamine membrane in L_β phase. Thereby, it is difficult to get information on the hydrocarbon chain tilt by this WAXS pattern. The electron density profiles and the WAXS patterns indicate that DHPC-MLV at pH 7.0 was in $L_\beta I$ phase and, at pH 1.5, was in $L_{\beta'}$ phase. Therefore, Fig. 1 shows that DHPC-MLVs at neutral pH (from pH 3.9 to 7.0) were in $L_\beta I$ phase, and those at low pH (from pH 1.2 to 2.2) were in $L_{\beta'}$ phase. DHPC-MLV at the intermediate pH (pH 2.3∼3.9) were in bilayer gel phase, which cannot be assigned to a more specific phase, such as $L_{\beta'}$ or $P_{\beta'}$ phase at present. A similar situation was reported in the trehalose-induced $L_\beta I$ to the bilayer gel phase in DHPC-MLV (Takahashi et al., 1997), in which the spacings were difficult to assign to the specific phase, such as $L_{\beta'}$ or $P_{\beta'}$ phase, at the intermediate concentration of trehalose (∼1.0 M). However, it is clearly evident

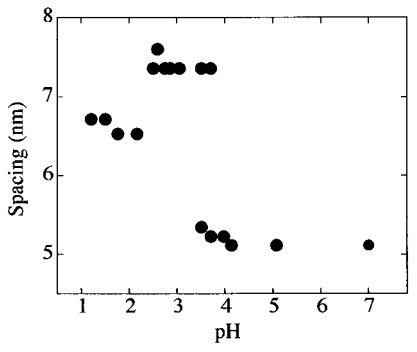

FIGURE 1 The spacing of DHPC-MLV at 20°C in various pH. The spacings were determined by the first diffraction peak of SAXS. Ionic strength of these various pH buffers was approximately 0.5 ($I \approx 0.5$).

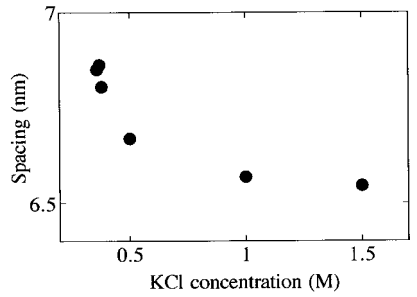

FIGURE 2 Dependence of KCl concentration on the spacing of DHPC-MLV at pH 1.5 at 20°C.

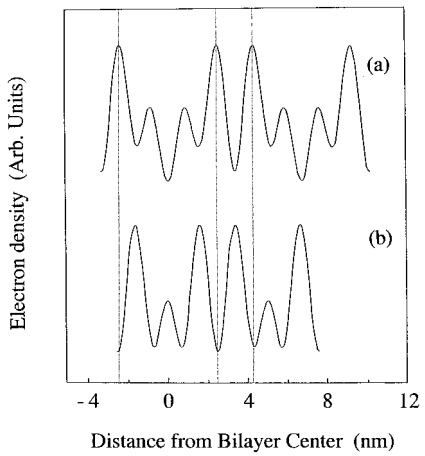

FIGURE 3 Electron density profiles for DHPC-MLV in (*a*) pH 1.5, (*b*) pH 7.0 at 20°C. Abscissa is a distance from bilayer center (nm). For each profile, the geometric center of the bilayer is placed at the origin of the abscissa. Low-density regions in the center of the profile correspond to the phospholipid hydrocarbon chains, and the high-density peaks on either side correspond to the lipid head groups.

that a phase transition from $L_\beta I$ phase to the bilayer gel phase in DHPC-MLV occurred at pH 3.9 with decreasing pH, and at low pH, <3.5, DHPC-MLVs were in bilayer gel phase.

pH Dependence of phase transition temperatures of DHPC-MLV

We have investigated the dependence of the phase transition temperature, T_m, from gel to liquid-crystalline phase of DHPC-MLV on pH by using a neutral fluorescent probe NPN (Träuble et al., 1976). Figure 4 shows temperature scans of the fluorescence intensity of NPN in the suspension of DHPC-MLV at pH 7.0 and 1.5. At pH 7.0, the fluorescence intensity increased abruptly at 43.1 and at 31.0°C. As indicated by Träuble, the increase in fluorescence intensity of NPN is due to the partitioning of NPN into the membrane, because the quantum yield of NPN in the membrane is higher than that in water (Träuble et al., 1976). Therefore, the increase at higher temperature is due to a phase transition of DHPC-MLV from a $P_{\beta'}$ phase to an L_α phase (T_m = 43.1°C). Similarly, the increase at lower temperature is due to a phase transition from an $L_\beta I$ to $P_{\beta'}$ phase (T_p = 31.0°C). These values are almost the same as those (T_m = 43.9°C and T_p = 33°C) determined by DSC. As shown in Fig. 4, DHPC-MLV at pH 1.5 had a higher transition temperature (T_m = 49.2°C) than that at pH 7.0, and also had no $L_\beta I$ to $P_{\beta'}$ phase transition. Figure 5 shows a pH dependence of phase transition temperatures at the same ionic strength ($I \approx 0.5$) as used in x-ray experiments. T_m increased with a decrease in pH when the pH value went below 5.0. Above 25°C, no $L_\beta I$ to $P_{\beta'}$ phase transition was detected below pH 3.5. Similar results were obtained by the light scattering experiments.

We have investigated a dependence of T_m on salt concentration at pH 1.5 (the positively charged state of DHPC) and pH 7.0 (the neutral state of DHPC) (Fig. 6). At pH 1.5, T_m increased with an increase in KCl concentration (C), linearly with $\sqrt{C}$. In contrast, at pH 7.0, T_m was almost the same irrespective of KCl concentration.

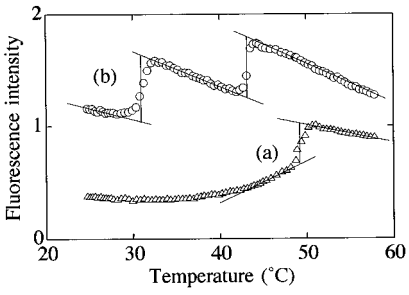

FIGURE 4 Temperature scans of the relative fluorescence intensity of NPN in suspension of DHPC-MLV at (*a*) pH 1.5 and (*b*) pH 7.0. $I \approx 0.5$. An abrupt increase in the fluorescence intensity indicates a phase transition. Heating rates were 0.5°C/min. A detail was described in the text.

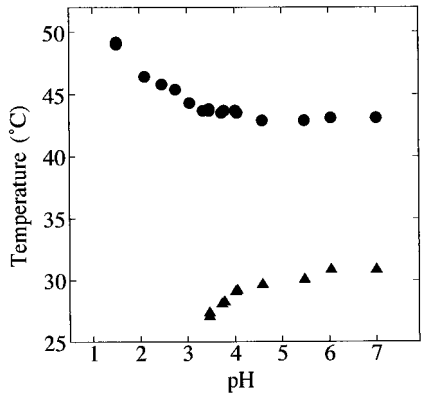

FIGURE 5 pH Dependence of phase transition temperatures of DHPC-MLV determined by the NPN fluorescence method. ●, gel to liquid-crystalline phase transition temperatures; ▲, $L_\beta I$ to $P_{\beta'}$ phase transition temperatures. Heating rates were 0.5°C/min. A detail was described in the text.

DISCUSSION

The mechanism of the low-pH induction of the phase transition from $L_\beta I$ to bilayer gel phase of DHPC membrane

Generally, the total chemical potential of the phospholipid in the membrane, μ, can be divided into three main contributions, i.e., $\mu = \mu_{hd} + \mu_{ch} + \mu_{th}$ (see e.g., Hatanaka et al., 1997). The term μ_{hd} is due to the membrane interface, μ_{ch} is a term due to the hydrophobic interior zone of the membrane, and μ_{th} is a term due to the interaction of the terminal methyl groups of the alkyl chains with surroundings. To elucidate the mechanism of the phase transition under discussion, it is worth considering the influence of pH on the chemical potential of DHPC molecules in the $L_\beta I$ phase (μ^{int}) and that in the bilayer gel phase (μ^{bil}). The difference of these chemical potentials, $\Delta\mu$, is expressed as

$$\begin{aligned}\Delta\mu &= \mu^{int} - \mu^{bil} \qquad (2)\\ &= (\mu^{int}_{hd} - \mu^{bil}_{hd}) + (\mu^{int}_{ch} - \mu^{bil}_{ch}) + (\mu^{int}_{th} - \mu^{bil}_{th})\\ &= \Delta\mu_{hd} + \Delta\mu_{ch} + \Delta\mu_{th}.\end{aligned}$$

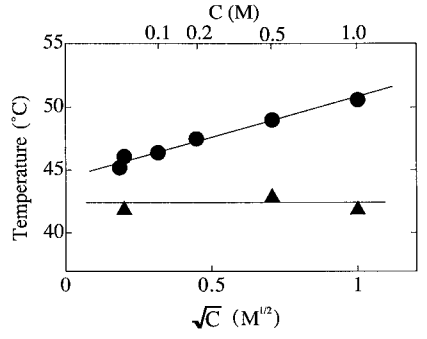

FIGURE 6 Dependence of the gel to liquid-crystalline phase transition temperatures of DHPC-MLV on KCl concentration (C[M]) or square root of KCl concentration ($\sqrt{C}$[M]): ●, in pH 1.5; ▲, in pH 7.0. The phase transition temperatures were determined by the NPN fluorescence method.

If $\Delta\mu$ is negative, i.e., $\Delta\mu = \Delta\mu(\mathrm{pH}) = \mu^{\mathrm{int}} - \mu^{\mathrm{bil}} < 0$, the $L_\beta I$ phase is energetically favorable for DHPC-MLV. In contrast, if $\Delta\mu > 0$, the bilayer gel phase is energetically favorable.

The terminal methyl groups are exposed to water in the $L_\beta I$ phase, and to the opposite monolayer in the bilayer gel phase. The contact between the segments of the alkyl chains and water is unfavorable because of the hydrophobic interaction (Tanford, 1991), which does not depend on pH. Thereby, the last term in Eq. 2 is always positive (i.e., $\Delta\mu_{\mathrm{th}} > 0$). The second term, $\Delta\mu_{\mathrm{ch}}$, is determined by the van der Waals interaction between the alkyl chains in the membrane (Simon and McIntosh, 1984), which is proportional to r^{-5} (where r is the separation between the alkyl chains) (Salem, 1962). Our x-ray diffraction data indicated that an average value of r in the $L_\beta I$ phase was smaller than that in $L_{\beta'}$ phase at pH 1.5 (see Results section). Taking into account that the concentrations of water and proton in the hydrophobic interior zone are significantly small in the gel phase, we can conclude that the van der Waals interaction between the alkyl chains does not depend on aqueous pH. Therefore, it is reasonable to think that $\Delta\mu_{\mathrm{ch}} < 0$ for any pH values and that values of $\Delta\mu_{\mathrm{ch}}$ do not depend on pH values. In contrast, the chemical potential of the membrane interface μ_{hd} is determined mainly by interactions between the headgroups of the phospholipids due to a steric hindrance and the effect of the interface hydration, and therefore, they depend significantly on the solution conditions. In summarizing all these contributions, we conclude that the only one term in Eq. 2 can depend on pH, is μ_{hd}.

Generally, μ_{hd} can be described as the sum of a term resulting from attractive interaction γA and that resulting from repulsive interaction R/A (Israelachvili et al., 1980; Cevc and Marsh, 1987; Marsh, 1996);

$$\mu_{\mathrm{hd}}(T, A) = \gamma(T)A + R(T)/A, \tag{3}$$

where R is a repulsive parameter and A is the area per lipid head. The main contribution to the attractive term (γA) is the hydrophobic interaction (Israelachvili et al., 1980; Marsh, 1996). As a value of γ, we use 39 mN m^{-1} in our present analysis, which Marsh (1996) has argued is realistic for the kind of models that we are considering here. The repulsive term (R/A) in Eq. 3 is determined mainly by the interaction between the head groups of the phospholipids resulting from the steric hindrance, an electrostatic interaction, and the interface hydration. The value of the repulsive parameter R can be estimated on the basis of the measurements of isothermal modulus of compression. Analyzing Fattal and Ben-Shaul's (1993, 1995) treatment of the lipid chain configuration at L_α, Marsh (1996) has concluded that R (L_α) $\approx 3.5 \times 10^{-36}$ mN m^3. This parameter at $L_{\beta'}$ phase, R ($L_{\beta'}$), becomes 2 $\sim$ 4 times larger than R (L_α), i.e., R ($L_{\beta'}$) $\approx (0.7 \sim 1.4) \times 10^{-35}$ mN m^3. Eq. 3 indicates that μ_{hd} (and, hence $\Delta\mu_{\mathrm{hd}}$) can be changed because of variations in either the attractive term γA or the repulsive term R/A. The physical origin of the attractive parameter γ is the hydrophobic interaction, which depends only weakly on aqueous pH. It is the reason why we select here the repulsive parameter R as the governing one. Moreover, the analysis of pH dependence of the gel to liquid-crystalline phase transition temperature shows that a change in pH value from 7.0 to 3.5 induces $\Delta R = -0.24 \times 10^{-36}$ mN m^3, and a change in pH value from 3.5 to 1.5 induces $\Delta R = -2.2 \times 10^{-36}$ mN m^3 (see Appendix).

Taking into account that $\mu_{\mathrm{th}}^{\mathrm{bil}} = 0$ and $\mu_{\mathrm{th}}^{\mathrm{int}} = \gamma_{\mathrm{th}} A$, we can now represent the chemical potentials μ^{int} and μ^{bil} as,

$$\mu^{\mathrm{int}}(R) = \mu_0^{\mathrm{int}} + \frac{R}{4A_{\mathrm{ch}}^{\mathrm{int}}} + \mu_{\mathrm{th}}^{\mathrm{int}} = \mu_0^{\mathrm{int}} + \frac{R}{4A_{\mathrm{ch}}^{\mathrm{int}}} + \gamma_{\mathrm{th}}(2A_{\mathrm{ch}}^{\mathrm{int}}), \tag{4}$$

$$\mu^{\mathrm{bil}}(R) = \mu_0^{\mathrm{bil}} + \frac{R}{2A_{\mathrm{ch}}^{\mathrm{bil}}} + \mu_{\mathrm{th}}^{\mathrm{bil}} \approx \mu_0^{\mathrm{bil}} + \frac{R}{2A_{\mathrm{ch}}^{\mathrm{bil}}}, \tag{5}$$

where μ_0^{bil} and μ_0^{int} are invariant parts of the total chemical potential at the bilayer gel phase and at the $L_\beta I$ phase, respectively. The areas per chain at both the phases are approximately the same $A_{\mathrm{ch}}^{\mathrm{int}} \approx A_{\mathrm{ch}}^{\mathrm{bil}} = A_{\mathrm{gel}}/2 \approx 0.2$ nm^2, judging from the WAXS patterns (see the Results section). Because the area per chain, A_{ch}, alone largely determines the invariant part of the chemical potential, $\mu_0^{\mathrm{bil}} \approx \mu_0^{\mathrm{int}} = \mu_0$. The surface density of the polar headgroups of lipids in the $L_\beta I$ phase is lower than that in the bilayer gel phase. This lowered density reduces the steric overlap of head group regions that consist of the hydrophilic segments and water molecules, and also increases conformational and mixing entropy of the head group regions. Therefore, the repulsive interaction free energy in the headgroup regions in $L_\beta I$ phase is lower than that in bilayer gel phase, which is expressed in Eqs. 4 and 5.

Figure 7 displays the influence of the repulsive parameter R on the chemical potential μ in the $L_\beta I$ phase (*curve 1*) and in the bilayer gel phase (*curve 2*). The curves were drawn on the basis of Eq. 4 and 5. There is a critical value of R, R^*,

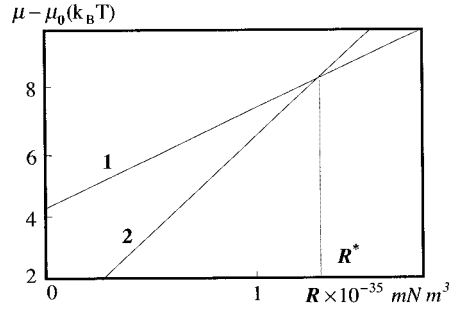

FIGURE 7 The dependence of the chemical potential μ of DHPC-MLV on the repulsive coefficient R. *Curve 1* corresponds to $L_\beta I$ phase; *curve 2* corresponds to bilayer gel phase. The critical value of R^*, where the phase transition from $L_\beta I$ to bilayer gel phase occurs, is $R^* = 1.3 \times 10^{-35}$ mN m^3. The curves have been drawn on the basis of Eqs. 4 and 5 for the following value of parameters: $\gamma = 39$ mN m^{-1}; $\gamma_{\mathrm{th}} = \gamma$; $A_{\mathrm{gel}} = 2A_{\mathrm{ch}}^{\mathrm{bil}} = 0.41$ nm^2. A detail was described in the text.

where $\mu^{int}(R^*) = \mu^{bil}(R^*)$. From Eqs. 4 and 5, R^* can be obtained as,

$$R^* = 8\gamma_{th}A_{ch}^2 = 2\gamma_{th}A^2. \quad (6)$$

Assuming $\gamma_{th} \approx \gamma$, and making use of the values of the parameters ($\gamma = 39$ mN m^{-1}; A = 0.41 nm^2), we obtain $R^* \approx 1.3 \times 10^{-35}$ mN m^3. This value of R^* is the same order as those of R estimated by Marsh. At large values of R where $R > R^*$ (i.e., large repulsion in the region of the polar headgroups), the energetically favorable phase of DHPC-MLV is the L_βI phase (*curve 1*). However, at small values of R where $R < R^*$, the bilayer gel phase becomes energetically favorable (*curve 2*).

Based on these considerations, we propose the mechanism of the low pH-induced phase transition of DHPC-MLV. At neutral pH, the repulsive parameter R is larger than its critical value R^*, e.g., $R_2 = R$ (pH 7.0) $= 13.24 \times 10^{-36}$ mN m$^3 > R^*$ (see Fig. 7). Moreover, this repulsion is so large that, contrary to the exposition of the terminal methyl groups in aqueous solution in L_βI phase, the energetically favorable phase of DHPC-MLV at pH 7.0 is the L_βI phase. Lowering pH from 7.0 to 3.5 changes the value of R by $\Delta R = -0.24 \times 10^{-36}$ mN m^3. At pH 3.5, R becomes equal to the critical value $R^* = R$ (pH 7.0) + $\Delta R = 13 \times 10^{-36}$ mN m^3, and the L_βI to bilayer gel phase transition occurs. Now the profit of head–head repulsive energy at the L_βI phase cannot compensate the energetic loss caused by exposition of the terminal methyl groups to aqueous solution at the L_βI phase. The bilayer gel phase of DHPC-MLV at low pH becomes energetically favorable. Therefore, the decrease of the repulsion between the polar headgroups induces the L_βI to bilayer gel phase transition.

Let us now consider the specific physical mechanism of the decrease in the repulsive parameter R at low pH. The headgroup of DHPC has one positive charge on a quaternary-amine group, N—$(CH_3)_3$, which is fully positive at any reasonable pH values. Its ionizable phosphate group PO_4 has intrinsic pK $\approx$ 1.5 (Tocanne and Teissie, 1990). Hence, at neutral pH, DHPC is at neutral zwitterion state. As pH of the solution decreases, the protonation of the phosphate groups increases. Consequently, at low pH, the surface of DHPC-MLV becomes positively charged. This protonation leads to two consequences: 1) the neutral zwitterion state of DHPC is transformed into a positively charged one; or 2) the interaction free energy of the DHPC's hydrophilic segment with water changes. The first effect leads to the appearance of the double electric layer in the solution, which increases the repulsion in the polar zone. Additionally, pure electrostatic repulsion between the headgroups itself also increases the repulsive parameter R. However, the second effect decreases R as follows. The negative charges of phosphate groups have a favorable interaction with water molecules (Port and Pullman, 1973; Frischelder et al., 1977; Cevc et al., 1981) and also contribute to the effective surface dipole density. Therefore, the protonation of the phosphate groups decreases the effective polarity of the membrane interface and decreases its interaction with water molecules, thereby decreasing the number of water molecules contained inside the hydrophilic head group region. As a result, the DHPC molecule tends to reduce its effective cross-sectional area per head group, or the conformational change of its hydrophilic segment may be induced (e.g., Bechinger and Seelig, 1991). It is also worth mentioning that an additional physicochemical factor decreases the repulsive parameter R at low pH. It is connected with the hydrogen bonding between adjacent phospholipid molecules. The phosphate groups at a nonionized state (i.e., at low pH) contain hydrogen bond donors as well as acceptors. Consequently, the intermolecular hydrogen bonding between the phosphate groups of adjacent phospholipid molecules can induce additional attraction between the headgroups. This can also reduce the repulsive parameter R at low pH.

Generally, the interplay between these two effects (i.e., pure electrostatic interaction between the head groups, and the variations of the interface hydration) can lead to an increase in the repulsion in some cases, and its decrease in others. The final result is determined by the prevailing effect in each specific case. Considering the above speculations, it follows that the effect of the variation of the interface hydration overcomes the pure electrostatic effect in the case of DHPC-MLV at low pH. As a result, the total repulsive interaction in the polar region decreases.

It is worth underlining that the mechanism of the low pH-induced phase transition of DHPC-MLV is almost the same as that of the phase transition of DHPC-MLV due to variation of poly(ethylene glycol) (PEG) concentration (Hatanaka et al., 1997). We reported that high concentrations of PEG − 6K [Mw = 7,500] induced a phase transition from the L_βI to the bilayer gel phase (Hatanaka et al., 1997), and explained it on the basis of the osmoelastic coupling theory (Yamazaki et al., 1989, 1992). The exclusion of PEG molecules from the region adjacent to the membrane surface induces an osmotic stress onto the membranes. To lower the chemical potential of water in the exclusion layer, the polar zone is compressed to produce elastic pressure (osmoelastic coupling). This compression induces the decrease of the repulsion between the DHPCs' headgroups.

Finally, it is interesting to compare the phase behavior of DHPC-MLV with that of DPPC-MLV. The structural difference between these phospholipids is that the DPPC molecule has two additional C═O groups. But, DPPC-MLV is in the bilayer gel phase ($L_{\beta'}$) in excess water at 20°C at neutral pH, whereas DHPC-MLV under the same conditions is in the L_βI phase. In accordance with the above conclusion, the repulsion in the polar headgroup region of the DHPC membrane is significantly larger than that of the DPPC one. Or, conversely, the attraction in the polar headgroup region of the DPPC membrane is significantly larger than that in the DHPC ones. As is known, the C═O group has a permanent electric dipole. Hence, these groups create some additional dipole–dipole interactions between adjacent phospholipid molecules in the polar region of the

DHPC membrane. It is reasonable to suggest that a conformation and an orientation of the adjacent phospholipid molecules have to be changed to minimize this dipole–dipole interaction energy. This means that the dipole–dipole interaction of the C═O groups create an additional attraction in the polar regions of the DPPC membrane, thereby decreasing the effective value of the repulsive parameter R. Another important factor has been indicated by Lewis et al. (1996). They have concluded from Fourier transform infrared spectroscopic measurements that the phosphate groups of DHPC-MLV in L_α phase were located in a more polar environment than those of DPPC-MLV in L_α phase. This result also supports our hypothesis that the interaction free energy of water with the headgroup segments of DHPC is lower than that of DPPC. Therefore, the repulsive parameter R of DHPC is larger than that of DPPC. Moreover, it is larger than its critical value R^* at neutral pH. In contrast, R of DPPC-MLV is less than the critical value R^* at neutral pH. This is why DPPC, under normal conditions, forms the bilayer noninterdigitated gel phase, but DHPC forms the interdigitated one. At present, the molecular origin of the difference between this interaction free energy of DHPC and that of DPPC is not understood. More analysis of the conformation of the headgroups (including glycerol backbone and C═O groups) or the structure of the interfaces of these PC membranes is necessary.

pH Dependence of the gel to liquid-crystalline phase transition temperature of DHPC-MLV

One more source of information about phase behavior of DHPC due to variation of pH is the variation of the temperature, T_m, of the gel to liquid-crystalline phase transition (Fig. 5). As is evident from Fig. 5, T_m of DHPC-MLV increases with a decrease in pH.

As we mentioned above, DHPC-MLV has the apparent positive surface electric charges at pH 1.5. The phase behavior of the charged phospholipid membrane has been explained by the theory based on the Gouy–Chapmann diffuse-double electric layer theory (e.g., Träuble et al., 1976, Jähnig et al., 1979). This shows that T_m decreases with an increase in the surface electric charge density σ, and also that at constant σ, T_m increases with an increase in the salt concentration, C, and its shift of T_m (ΔT_m) is proportional to $\sqrt{C}$. Comparing the results of Fig. 6 with this classical theory, one can conclude that the behavior of the charged DHPC-MLV is totally in compliance with this classical theory. In contrast, according to this theory, the transformation of DHPC from a neutral state to a charged one will decrease T_m. As pH decreases, the surface charge density σ of DHPC increases. Hence, the effect of electrostatic interaction explains the increase of T_m with an increase in salt concentration, but cannot explain the increase of T_m with a decrease in pH. Therefore, we can conclude that our experimental results contradict this theory.

To explain this contradiction, it is necessary to take into account that the transition temperature T_m is determined by the 2D lateral pressure in the region of alkyl chains of the membrane Π_{chain} (Nagle, 1980; Cevc and Marsh, 1987), moreover $T_m \propto \Pi_{chain}$. The mechanical equilibrium of lipid membrane is provided by the balance of three kinds of lateral pressures (Israelachvili, 1992; Kinoshita et al., 1998: 1) the lateral pressure in the polar head region of membrane Π_{head}; 2) the effective attractive interfacial pressure resulting from the hydrophobic interaction between the alkyl chains and water at the membrane surface γ; 3) the lateral pressure in the hydrophobic chain region of the membrane Π_{chain}. The balance equation is

$$\Pi_{chain} = \gamma - \Pi_{head}. \quad (7)$$

As we discussed before, γ does not depend on pH. Hence, the decrease of Π_{head} leads to an increase in Π_{chain} and vice versa. Because $T_m \propto (\gamma - \Pi_{head})$, the result of Fig. 5 demonstrates that the lowering of pH decreases Π_{head}. Hence, this also indicates that the repulsive force between the head groups of DHPC decreases with a decrease in pH. A specific physical mechanism of the variation of Π_{head} is the same as we discussed above. The increase in the repulsion between the charged headgroups of DHPC membranes at low pH decreases the lateral pressure in the hydrophobic chain region Π_{chain}, which induces a decrease in T_m. However, at the same time, the lowering of pH changes the interfacial hydration, which decreases Π_{head}, and thereby, increases Π_{chain}. The latter effect prevails on the electrostatic effect, and hence, T_m increases as pH decreases.

CONCLUSION

We have investigated the influence of pH on the structure and phase behavior of DHPC-MLV. The results of x-ray diffraction experiments clearly demonstrate that the $L_\beta I$ to bilayer gel phase transition occurred in DHPC-MLV at pH 3.9. Moreover, at low pH (<3.5), DHPC-MLVs were in the bilayer gel phase. We also observed that T_m of DHPC-MLV increased as pH decreased.

Our thermodynamic analysis indicates that the main factor of the low pH-induced $L_\beta I$ to bilayer gel phase transition is the decrease of the repulsive interaction between the head groups of DHPC membranes. At low pH, due to the protonation of the phosphate group, the positive surface charges of this membrane increase the electrostatic repulsion between the headgroups. However, at the same time, there is an increase in the interaction free energy between the hydrophilic segments and water, which decreases the repulsive interaction between the headgroups. The latter effect dominates the former one, and thereby, the total repulsive interaction in the interface of this membrane decreases. It also increases the lateral compression pressure of the membrane, resulting in the increase in T_m. The decrease in the repulsive interaction due to the protonation of the phosphate group at low pH highlights the critical role of the interfacial region as a determinant of the structure and organization of phospholipid membranes.

APPENDIX: VARIATION OF THE REPULSION PARAMETER *R* DUE TO VARIATION OF pH

Träuble et al. (1976) have introduced the expression for the shift of the temperature of the main transition T_m of the lipid bilayer resulting from the variations of the electrostatic term of free energy. Extending their method, one can easily obtain a general expression for the shift of T_m (ΔT_m) resulting from any external effects on the lipid membrane,

$$\Delta T_m = T_m - T_m^* = \frac{\mu_{liq}^{var} - \mu_{gel}^{var}}{\Delta S} = \frac{\Delta \mu^{var}}{\Delta S^*}, \qquad (A1)$$

where T_m^* is the temperature of main phase transition (bilayer gel $\Leftrightarrow L_\alpha$) at the standard conditions without external effects (i.e., in our case, at pH 7.0); T_m is the phase transition temperature upon external effects (in our case, at any pH other than 7.0); μ^{var} is the variation of the chemical potential resulting from change of pH; ΔS^* is the entropy difference between gel and fluid states of the lipid membrane under the standard conditions, i.e., $\Delta S^* = S_{liq}$ (pH 7.0) $- S_{gel}$ (pH 7.0). From Eq. A1, one can obtain $\Delta \mu^{var} = \Delta T_m \Delta S^* = 0.040 \Delta T_m (k_B T)$, when we use $\Delta S^* = 25.2$ (cal/mol deg.) $= 0.040\ k_B T$/deg for DHPC-MLV (see Kim et al., 1987).

Then, assuming the variation of pH mainly affects the repulsive parameter R (i.e., $\mu_{liq}^{var} = \Delta R/A_{liq}$ and $\mu_{gel}^{var} = \Delta R/A_{gel}$, see discussion on this subject in the main text),

$$\Delta R = \Delta \mu^{var} \frac{A_{gel} A_{liq}}{A_{liq} - A_{gel}} = \Delta T_m \Delta S^* \frac{A_{gel} A_{liq}}{A_{liq} - A_{gel}}. \qquad (A2)$$

Judging from the results of Fig. 5, a change in pH value from 7.0 to 3.5, where the $L_\beta I$ to bilayer gel phase transition occurs, increases T_m by 0.6°C. Using Eq. A2, a change of the repulsive parameter during this pH change is calculated as $\Delta R = R$ (pH 7.0) $- R$ (pH 3.5) $= -5.4 \times 10^{-20}\ k_B T\ m^2 = -0.24 \times 10^{-36}$ mN m^3 (where we used parameters for DHPC: $A_{liq} = 0.61$ nm^2 and $A_{gel} = 0.48$ nm^2, $\Delta S^* = 25.2$ (cal/mol deg.) $= 0.040\ k_B T$/deg, see Kim et al., 1987). At the same time, a change in pH value from 3.5 to 1.5 increases T_m by 5.5°C. Therefore, $\Delta R = R$ (pH 3.5) $- R$ (pH 1.5) $= -50 \times 10^{-20}\ k_B T\ m^2 = -2.2 \times 10^{-36}$ mN m^3.

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