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PROTON-INDUCED PHASE SEPARATION IN PHOSPHATIDYLSERINE/PHOSPHATIDYLCHOLINE MEMBRANES

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Summary

Effects of pH and ionic strength on phosphatidylserine/phosphatidylcholine mixed membranes prepared on Millipore filter pore surfaces have been studied using spin-labeled phosphatidylcholine. Lowering pH at constant ionic strength and lowering ionic strength at constant pH caused a lateral reorganization of the membrane. The trigger was protonation of the serine carboxyl group which caused solidification of phosphatidylserine molecules in the membrane, leaving a fluid phase consisting mainly of phosphatidylcholine. The apparent p*K* for the proton-induced phase separation was measured in a wide range of salt concentrations. The ionic strength dependence was satisfactorily explained based on the electrostatic free energy of proton in the field of membrane surface potential. The Gouy-Chapman theory gave a good approximation for the surface potential. The surface p*K* of phosphatidylserine and phosphatidic acid vesicles was directly measured in various salt concentrations by ^{31}P-NMR and the results confirmed validity of the Gouy-Chapman-type analysis. The lateral reorganization was triggered by electrostatic interaction but the bulk of the stabilization energy for the structural change would be the gains in intermolecular van der Waals energy due to closer packing of phosphatidylserine on solidification.

Introduction

Phase transitions and separations lead to reorganization of membrane intrinsic components [1], enhancement of permeability and reactivity in phospho-

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lipid vesicles and biological membranes [2—4], and fusion of phospholipid vesicles [5,6]. Phase separations can be induced thermally in phospholipid mixtures under suitable immiscibility conditions of acyl chains [7—9], or triggered isothermally by charged substrates in mixed phospholipid membranes with different charges. The Ca^{2+}-induced phase separations have been studied extensively by spin-label technique [10—13], differential scanning calorimetry [14,15], and freeze-fracture electron microscopy [15]. Basic proteins also caused similar phase separations [16—18]. The ionotropic phase separations may be biologically more significant than the thermotropic ones because of isothermal induction and regulation. The importance of the electrostatic regulation of structural changes in charged phospholipid membranes has been pointed out by Träuble [19].

We have recently found that proton caused disappearance of the Ca^{2+}-induced phase separation in phosphatidylserine/phosphatidylcholine mixed membranes [20]. Proton competed with Ca^{2+} for binding to the serine head group and, as a result of the protonation, caused phase separation in the mixed membrane. The present paper describes details of the proton-induced phase separation. Dependence of the phase separation on pH and ionic strength of the bathing medium was explained on the basis of Gouy-Chapman theory.

Materials and Methods

Phosphatidylserine (bovine brain), phosphatidylcholine (egg yolk), and spin-labeled phosphatidylcholine with 12-nitroxide stearate at 2-position were prepared as described previously [20]. Phosphatidic acid was obtained by the action of cabbage phospholipase D on egg yolk phosphatidylcholine [21] and then converted to monopotassium salt.

Phospholipid multilayered membranes were prepared in a Millipore filter with average pore diameter of 5 μm as described previously [20]. The composition was phosphatidylserine/spin-labeled phosphatidylcholine at a molar ratio of 9 : 1 in most cases. The bathing media always contained 0.05 mM EDTA and were buffered with acetate, phosphate, or borate, depending on the pH desired. The ionic strength was adjusted with KCl. ESR spectra were measured at room temperature (22° C) with an X-band spectrometer (JEOLCO Model ME-2X). For NMR measurements, single-bilayered phospholipid vesicles were prepared by sonicating at 0° C in 0.1 mM EDTA and various concentrations of KCl. The pH was adjusted by adding HCl or KOH. The phospholipid bilayer was found to be a good insulator to H^+. When KOH was added to the sonicated dispersion, the ^{31}P-NMR peak split into two lines: one at the initial position and the other at a lower frequency position. The split lines were stable for at least a few hours at room temperature. The shifted peak must arise from the phosphate group on the outer surface of vesicles and the chemical shift of the peak was plotted as a function of the external pH. NMR spectra were measured at 30° C with a JNM PFT-100 spectrometer operating at 40.48 MHz with a broad-band proton decoupling. 200—500 of free induction decays were accumulated using 45° pulse. A 1 M ^2H_2O solution of pyrophosphate in a central capillary was used as an external standard.

Results

Induction of phase separation by lowering pH

The medium pH gave a marked effect on the mixed phospholipid membrane. Fig. 1 shows ESR spectra of phosphatidylserine/spin-labeled phosphatidylcholine membranes equilibrated at various pH values in 100 mM KCl. The spectra at lower pH values (Fig. 1a—c) were markedly broadened due to spin-spin interactions, indicating clustering of spin-labeled phosphatidylcholine in the membrane. The spectral change was quite similar to that induced by Ca^{2+} (dotted spectrum in Fig. 1).

For quantitative presentation of the broadening, a parameter α defined as (1/2)(AB/CD) (see Fig. 1) was used as in the previous study [20]. The smaller values of α represent more broadening and stronger spin-spin interactions and correspond to more extensive clustering. The spectral parameter was obtained at various pH values and plotted against pH in Fig. 2. The broadening started to occur at pH 4.8 and stopped at 2.5. The apparent pK was read as 4.2 from the midpoint.

The bovine phosphatidylserine membrane is known to become solid on lowering pH to approx. 2 at room temperature [13,14]. In other words, the thermotropic phase transition temperature became higher at lower pH values; 6°C at pH 7.4 and 26°C at pH 2.5 [14]. The pH dependence was attributed to protonation of the serine carboxyl group of phospholipid in the membrane with pK of 4.4 in 100 mM NaCl [15]. Therefore, the phase separation observed

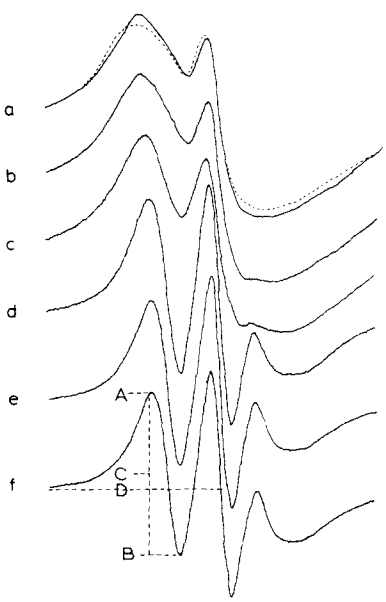

Fig. 1. ESR spectra of phosphatidylserine/spin-labeled phosphatidylcholine (9 : 1) membrane at various pH values in 100 mM KCl: (a) 1.8; (b) 2.5; (c) 3.5; (d) 4.5; (e) 5.5, and (f) 7.5. The bathing medium contained 0.05 mM EDTA and was buffered with 10 mM phosphate, acetate, or borate. Spectra were measured at 22°C after soaking the membrane overnight in the medium. ... in (a) is the spectrum obtained when the membrane was soaked in 10 mM $CaCl_2$. The peak heights in (f) are shown for definition of α = [(1/2) (AB/CD)].

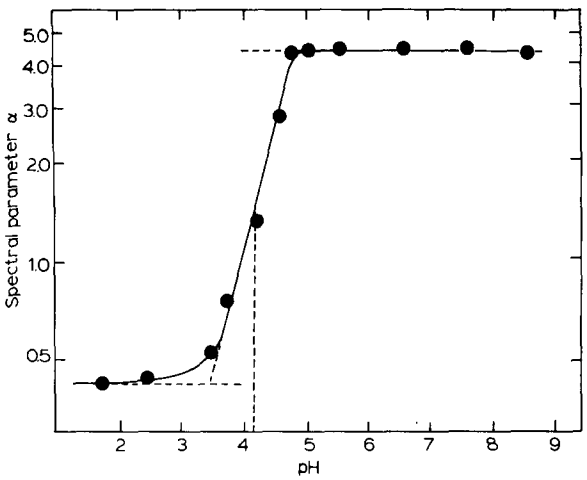

Fig. 2. The spectral parameter α against pH in 100 mM KCl. The parameter was calculated with the spectra measured at various pH values in 100 mM KCl (see Fig. 1).

on lowering pH is due to protonation of the serine carboxyl group in the mixed membrane which lead to solidification and segregation of phosphatidylserine molecules in the membrane, accompanied by clustering of spin-labeled phosphatidylcholine.

The ESR spectra with α value larger than approx. 1 were similar to those of homogeneous mixtures of phosphatidylserine/spin-labeled phosphatidylcholine at appropriate molar ratios. By using an empirical relationship between log α and the molar ratio [20], we may calculate concentration of spin-labeled phosphatidylcholine in the fluid phase under phase separation. For example, the α value 1.38 at pH 4.1 corresponds to the spin label concentration of 17%. Since the initial spin label concentration was 10%, the fraction of phospholipids in the fluid phase is given to be 10/17 = 0.59. The fraction of phospholipids in the solid phase is therefore 1 − 0.59 = 0.41. On the other hand, the spectra with α value smaller than 1 had some different features from those of homogeneous mixtures and can be taken as superposition of variously broadened spectra. The spectra can be interpreted to be due to formation of small patches of spin-labeled phosphatidylcholine with various sizes as in the case of Ca^{2+}-induced phase separations [10,11].

When the mixed membranes containing less phosphatidylserine were used, the spectra in the whole pH range appeared homogeneous and the pH titration curve for the fraction of phospholipids in the fluid phase was obtained from the α value. For example, for phosphatidylserine/phosphatidylcholine/spin-labeled phosphatidylcholine (0.67 : 0.25 : 0.08) membrane, the α value changed from 8.0 to 1.3 in a pH range from 6.2 to 2.7 in 10 mM KCl. The fraction of phospholipid in the fluid phase reached 0.45 in the lower pH limit. This value became somewhat larger (0.55) at a higher temperature (40°C) [22].

The proton-induced phase separation occurred more quickly at lower pH values. The separation became equilibrated in 1 h at pH 1.8 and 2 h at pH 2.5,

while it was not equilibrated after 2 h at pH 3.5. The reverse reaction was rapid and the spectrum was restored in 10—20 min when the membrane was soaked in 100 mM KCl, pH 7.4.

Induction of phase separation by lowering ionic strength

The phase separation was also observed when the salt concentration of the bathing medium was reduced. Fig. 3 shows ESR spectra of the mixed membrane soaked in various concentrations of KCl at a constant pH 5.0. The spectra in low ionic strength media were exchange broadened owing to clustering of spin-labeled phosphatidylcholine in the membrane. The phase separation occurred slowly and required almost a day for equilibrium. The reverse reaction was faster and took 0.5 h in 100 mM KCl.

Phase separation as a function of pH and ionic strength

ESR spectra of the mixed phospholipid membrane were measured with changing pH in various concentrations of KCl. The spectral parameter was obtained and plotted as a function of pH and ionic strength in Fig. 4. The results clearly indicate that the titration curve was shifted to higher pH on lowering salt concentration; pK for the phase separation was increased on decreasing ionic strength.

The apparent pK for the phase separation was plotted against the log of salt concentration (Fig. 5a). The data points were on a straight line except for higher salt concentrations. The linear relationship can be explained based on

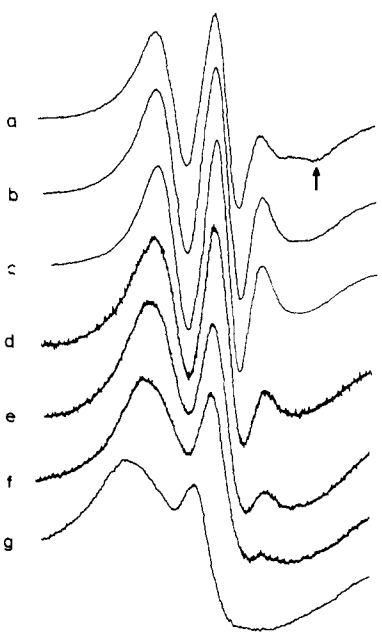

Fig. 3. ESR spectra of phosphatidylserine/spin-labeled phosphatidylcholine (9 : 1) membrane in various concentrations of KCl at pH 5.0: (a) 3 M; (b) 1 M; (c) 100 mM; (d) 25 mM; (e) 10 mM; (f) 5 mM, and (g) 1 mM. The medium contained 0.05 mM EDTA and was buffered with acetate at concentrations of about 1/10 of that of KCl. Spectra were measured after overnight soaking of the membrane.

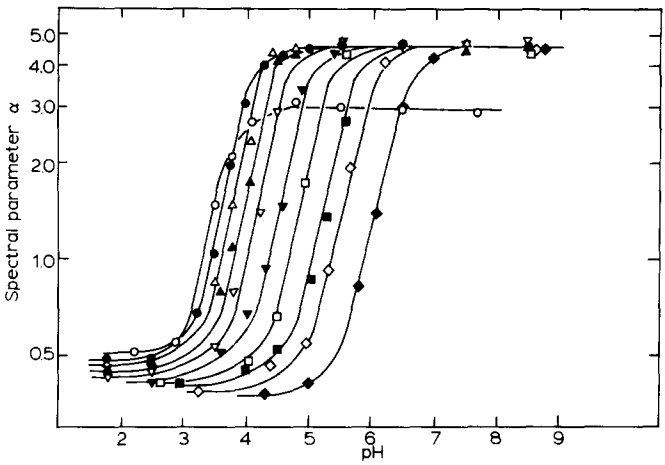

Fig. 4. The spectral parameter α against pH in various concentrations of KCl: ○, 3 M; ●, 1 M; △, 500 mM; ▲, 250 mM; ▽, 100 mM; ▼, 50 mM; □, 25 mM; ■, 10 mM; ◇, 5 mM, and ◆, 1 mM.

the electrostatic free energy of proton in the field of membrane surface potential. Assuming Gouy-Chapman theory for the surface potential [23] and Boltzman distribution for the proton concentration, and taking high-potential approximation, pK of the surface acidic group, pK_s, in monovalent electrolyte is given by

$$pK_s = pK_0 + 0.58 - \log n \qquad (1)$$

where pK_0 is the pK in bulk and n the salt concentration. The surface area per phospholipid was taken as 65 Å^2 [24]. The straight line in Fig. 5a was drawn

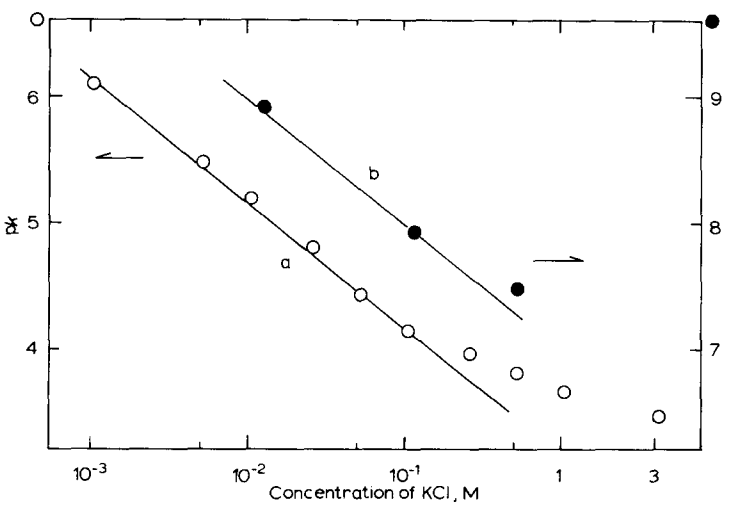

Fig. 5. (a) Apparent pK for the proton-induced phase separation against salt concentration. (b) Surface pK of sonicated phosphatidic acid vesicles against salt concentration. The straight lines are drawn by Eqns. 1 and 2 based on Gouy-Chapman theory.

according to Eqn. 1 with the pK_0 chosen as 2.62 to make a best fit to the experimental data. The pK_0 agrees well with pK of the carboxyl group of a water-soluble phosphoserine, 2.65 [25]. The deviation at higher salt concentrations is due to invalidity of high-potential approximation in the region.

The titration curve in the highest concentration of KCl (3 M) was somewhat different from the others in that the α values at higher pH values were smaller (Fig. 4). This is not due to persistence of the phase separation in those pH values but due to different ESR spectral shape as can be seen by the bump at higher field position (see arrow in Fig. 3a). The difference can be more clearly observed with the mixed phospholipid membrane containing smaller concentrations of spin label. The overall splitting value in 3 M KCl (43 G) was larger than that in more dilute KCl solutions (40 G), indicating reduced flexibility of phospholipid alkyl chains in the high-salt medium.

Direct measurement of surface pK by ^{31}P-NMR

An attempt was made to measure the surface pK of phosphatidylserine membrane directly by ^{31}P-NMR to confirm the validity of Gouy-Chapman-type analysis. Fig. 6A shows chemical shift of ^{31}P resonance peak of phosphatidylserine vesicles as a function of pH. Unfortunately, the full titration curve was not obtained because of precipitations below pH 2.3. However, it is inferred from the data that pK for the phosphate group of phospholipid was smaller than 3.6 in 100 mM KCl. The acidic site responsible for the proton-induced phase separation is therefore probably the serine carboxyl group.

We then measured the surface pK of phosphatidic acid vesicles in the second ionization region where no precipitation occurred. Fig. 6B shows ^{31}P chemical shift data as a function of pH in various salt concentrations. Decreasing salt concentration shifted the titration curve towards higher pH. The surface pK was plotted against log of salt concentration in Fig. 5b (filled circles). A linear

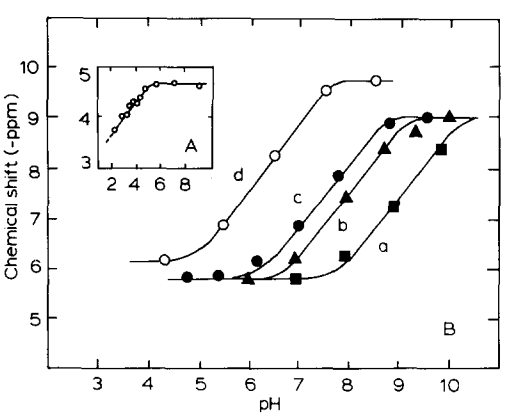

Fig. 6. ^{31}P-NMR chemical shift of sonicated vesicles of phosphatidylserine (A) and phosphatidic acid (B) against external pH in various salt concentrations. Concentration of KCl added was 100 mM (A), 0 mM (Ba), 100 mM (Bb), and 500 mM (Bc). (Bd) The titration curve for glycerophosphate in 100 mM KCl and 0.1 mM EDTA. Concentrations of phosphatidic acid monopotassium salt and phosphatidylserine were 25 mM.

relationship was observed again. The straight line was drawn by

$$pK_s = pK_0 + 1.66 - \log n \quad (2)$$

where pK_0 was chosen as 5.60 for the best fit of the data. The pK of a water-soluble glycerophosphate was 6.1 by the NMR measurement (Fig. 6Bd) and 6.43 by the literature [26]. The discrepancy between the chosen pK_0 and the solution pK is somewhat larger in this case, which may be due to abnormality of the highly curved vesicles.

Discussion

The lateral organization of mixed phosphatidylserine/phosphatidylcholine membranes was modified depending on the pH and ionic strength of the bathing medium. Phosphatidylserine molecules formed solid aggregates and separated from the fluid phase consisting mainly of phosphatidylcholine at lower pH or at lower ionic strength. The trigger was the protonation of phosphatidylserine carboxyl group. The pH and ionic strength dependence was satisfactorily explained by the electrostatic free energy of proton in the field of surface potential. The Gouy-Chapman theory was a good approximation for the surface potential despite all its known simplifications. The validity was also directly shown by ^{31}P-NMR of charged phospholipid vesicles.

Most of the stabilization energy for the proton-induced phase separation probably come from the gains in the intermolecular interaction energy due to closer packing of phosphatidylserine on crystallization; gains in the van der Waals interaction between the alkyl chains, between the polar head groups, and possibly by hydrogen bonding between the serine head groups. Decreased electrostatic repulsion due to the charge neutralization may also contribute to the stabilization. Träuble has shown that the charge neutralization alone may cause phase separations under suitable condition [19]. However, the electrostatic effect would not contribute much to the stability for the phase separation in the present system. The electrostatic contribution was calculated as 0.35 kcal/mol at maximum when the molecular area was assumed to be unchanged on protonation [19]. On the other hand, the enthalpy change at the phase transition for various synthetic and natural phospholipid membranes ranged around 5 kcal/mol. The enthalpy change of bovine brain phosphatidylserine was reported as 4.5 kcal/mol, irrespective of pH [14], and that of dimyristoyl phosphatidylserine at pH 6 to be approx. 8 kcal/mol [15]. It is interesting to compare the transition temperatures for dimyristoyl phosphatidylcholine and dimyristoyl phosphatidylserine neutral form, 23° C and 54.6° C, respectively [15]. If the entropy change at the transition were largely due to long alkyl chains, the temperature difference would indicate contribution from the serine head group to the enthalpy change.

The lateral organization change in the mixed phospholipid membrane was triggered by protonation or electrostatic interaction but the bulk of the energy change involved was in the hydrophobic van der Waals interaction. Such electrostatic regulations of structural changes may be an interesting common pattern in biomolecules and biological structures. Since the cell surfaces are heterogeneous and ionic environments may be locally different and change-

able, and since Ca^{2+} and H^+ are the competing ions for a phospholipid head group, the lateral reorganization of phospholipids in biological membranes may be of crucial physiological significance.

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