Microdomain Formation in Phosphatidylethanolamine Bilayers Detected by ^2H NMR

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In deuterium NMR spectra of phosphatidylethanolamine bilayers with an extremely high content of saturated fatty acids, each C1 deuteron of the glycerol backbone gave rise to a doublet.[1] This suggests the presence of two backbone conformations, the exchange between which is slow on an NMR time scale. The origin of the two conformations has been investigated in this work using saturated 1,2-diacyl-*sn*-glycero-3-phosphoethanolamine specifically deuterated in the glycerol backbone. The results showed that the two conformations originate from different domains, which have different fatty acid compositions. The differential scanning calorimetry of the bilayers suggested that the size of the domain is not large enough to show an independent phase transition. Thus, the formation of microdomains in the phosphatidylethanolamine bilayers has been concluded. Conformational difference in different domains was shown to be restricted to the C1 position of the glycerol backbone. The microdomains of phosphatidylethanolamine were retained even in the presence of other phospholipids.

Introduction

Phospholipids are important constituents of biological membranes. Since knowledge of the structure and dynamics of phospholipids in bilayers is essential for understanding of their functional roles, their physicochemical properties have been studied extensively. Deuterium magnetic resonance (^2H NMR) has been widely used to investigate the conformation and dynamics of lipid molecules in bilayers.[2,3]

Phosphatidylethanolamine is one of the major phospholipids in biomembranes. Although phosphatidylethanolamine (PE) and phosphatidylcholine (PC) are the two major zwitterionic phospholipids, their characteristics are very different. PE shows a transition from the lamellar to hexagonal II phases, while PC does not. PE is mainly located in the inner leaflets of mammalian plasma membranes, while PC is mainly located in their outer leaflets. It has been also reported that membranes containing PE rather than PC as the zwitterionic phospholipid showed great selectivity in association with protein kinase C and other cytoplasmic proteins.[4] PE was also shown to enhance rhodopsin photochemical activity in the reconstituted system.[5] Physicochemical basis of specificity of PE is not yet clear. Especially, there are few pieces of information on the nature of the intermolecular interactions among PE molecules. Such properties would play an important role in the interaction with membrane proteins and other phospholipids. Furthermore, phosphatidylethanolamine liposomes are now investigated for the purpose of drug delivery, gene transfer and immunodiagnostic application.[6] Physicochemical basis of the specificity of PE would also provide us with the principles to design such liposomes.

Yoshikawa et al. found out that each deuteron at the C1 position of the glycerol backbone of phosphatidylethanolamine in *E. coli* lipid bilayers with an extremely high content of saturated fatty acids gives rise to two different quadrupole splittings.[1] The two quadrupole splittings are the evidence for the presence of two different conformations at the C1 position of the glycerol backbone of phosphatidylethanolamine in the bilayers, and the exchange rate between the two conformations being slower than the NMR time scale. This can provide a clue to elucidate the microscopic intermolecular interactions of PE. Two possible explanations were suggested for the appearance of the two quadrupole splittings.[1] One was the formation of a bimolecular complex, which has two different glycerol conformations. The other was the formation of at least two kinds of domains, which have different glycerol conformations. These domains should have different unsaturated fatty acid contents, which should affect the conformation of the glycerol backbone. To examine these possibilities, we have synthesized 1,2-dipalmitoyl- and 1,2-dimyristoyl-*sn*-glycero-3-phosphoethanolamine and performed the ^2H NMR measurement of their bilayers in this work. In addition to it, the effect of other phospholipids on the two conformations of the phosphatidylethanolamine has been investigated.

Materials and Methods

Synthesis of [$^2H_5$]-glycerol and [2-$^2H_1$]-glycerol. Perdeuterated glycerol ([$^2H_5$]-glycerol) was synthesized by reduction of diethylketomalonate (1.83 mL) with $LiAl^2H_4$ (1 g) in tetrahydrofuran (THF, 45 mL). The reaction mixture was refluxed for about 1 hr in an ice bath under nitrogen gas flow. Then, 10 mL of water was added for inactivation of $LiAl^2H_4$. After filtration, THF was removed by evaporation. The obtained aqueous solution was applied to an ion exchange column (Dowex 50W-X8) to remove $A1^{3+}$ ions. [2-$^2H_1$]-glycerol was obtained by reduction of dihydroxyacetone (4.39 g) with NaB^2H_4 (1 g) in ^2H_2O (30 mL).[1]

Growth of a glycerol-requiring mutant of *E. coli*. An auxotroph (*E. coli* K-12 UFA^{ts}GRA) requiring unsaturated

fatty acids (temperature-sensitive) and glycerol[1] was grown at 28 °C in a basal medium containing the following (per liter); K_2HPO_4, 7 g; KH_2PO_4, 2 g; Na_3 citrate, 0.57 g; $(NH_4)_2SO_4$, 1 g; $MgSO_4 \cdot 7H_2O$, 0.1 g; vitamin B_1, 1 mg; glycerol, 2.5 g (deuterated glycerol, 0.1 g); casamino acids (Difco, vitamin free), 10 g. The auxotroph requires unsaturated fatty acids for growth at 42 °C but can grow in the basal medium containing 2.0% KCl without supplementation of unsaturated fatty acids at 42 °C. Such a culture accumulates saturated fatty acids at extremely high content in the phospholipids (*ca.* 90%) of the cell membranes.[7] The cells were cultured up to the end point of the logarithmic phase (about 14 hr at 42 °C)in a 10-litter bottle with air bubbling and then harvested by centrifugation. Phosphatidylethanolamine, phosphatidylglycerol and cardiolipin were purified from the cells grown at 42 °C.[1]

Preparation of phospholipids. Phospholipids were extracted from the cells according to the method of Bligh and Dyer,[8] and then further purified by silicic acid column chromatography. The total phospholipid extract (250-300 mg) was applied to a silicic acid column (30×300 mm), which was then eluted with chloroform/methanol/water (65 : 25 : 4, v/v). A Yamazen fast flow liquid chromatography system was used for the purification with the flow rate of 0.6 mL/min. Each phospholipid species were identified by silicic acid thin layer chromatography. The percentage of deuteration of the glycerol backbone was estimated from a ^1H NMR spectrum of deuterated phosphatidylethanolamine.[1] The extent of deuteration was about 70% in average. ^1H NMR spectra were obtained with a Bruker AM400 NMR spectrometer.

Saturated phosphatidylethanolamine was synthesized according to the reported method.[9] 1,2-dimyristoyl- and 1,2-dipalmitoyl-*sn*-glycero-3-phosphoethanolamine (DMPE and DPPE, respectively, hereafter) were prepared from *E. coli* phosphatidylethanolamine and fatty acid anhydride, using polymer-supported trityl as a protective group for the amino group of phosphatidylethanolamine. The solid-phase synthesis makes the separation of the intermediate products much easier. Each reaction step was confirmed by monitoring the C=O stretching vibration band at 1740 cm^{-1} in an infrared spectrum. Yield of DMPF and DPPF was 71%. Specific deuteration in the glycerol backbone was performed by using specifically deuterated *E. coli* phosphatidylethanolamine. Purity was checked by silicic acid thin layer chromatography and differential scanning calorimetry. Infrared spectra were obtained with a Shimadzu FTIR-4000 spectrophotometer.

^2H NMR measurements. ^2H NMR spectra were obtained with a JEOL FX-200 NMR spectrometer equipped with a solid-state system, and Bruker MSL-300 and AM-400, and Chemagnetics CMX-400 NMR spectrometers. In the case of AM-400, a home-built high power probehead, in which the coil was designed to accommodate a vertical sample tube, was used. Phospholipids were dispersed at 55 °C (at 75 °C for DPPE) in a solution of 0.1 M PIPES buffer (pH 7.2) containing 2 mM EDTA, and then centrifuged to obtain a pellet. Deuterium-depleted water (less than 0.2 ppm, purchased from Commissariat a L'Energie Atomique) was used for all sample preparations to eliminate the natural abundance deuterium signal of water, ^2H NMR spectra of phospholipid dispersions (multilamellar liposomes) were obtained by the quadrupole echo technique with a spectral width of 100 kHz and a recycle time of 0.5 s. ^{31}P NMR spectra were measured with a CMX400 NMR spectrometer under proton decoupling.

Differential scanning calorimetry. Thermograms of phospholipid dispersions were obtained by a Privalov-type calorimeter, DASM-4.

The scan rate was 0.5 K/min. The phospholipid concentration was 10 mg/mL in 0.1 M PIPES, 2 mM EDTA (pH 7.2).

Results

Yoshikawa *et al.* reported ^2H NMR spectra of a phospholipid mixture obtained from *E. coli.*[1] The signal of each C1 deuteron of the glycerol backbone of phosphatidylethanolamine (PE) in the mixture was found to split to a doublet. Figure 1A is a ^2H NMR spectrum of bilayers of phosphatidylethanolamine purified from the *E. coli* phospholipid mixture mentioned above. The assignments were given according to the reported ones.[10] 1S, 1R, 2, 3S and 3R stand for the deuterons indicated in the structure on the top of the figure with

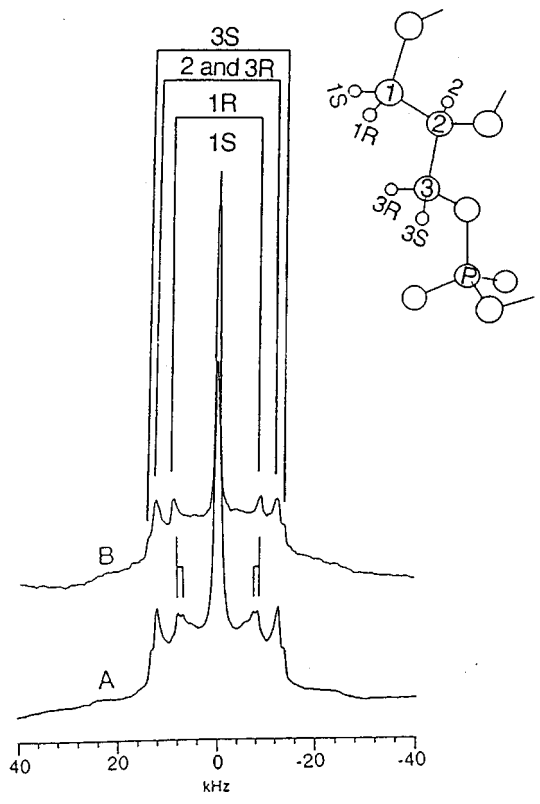

Figure 1. ^2H NMR spectrum of *E. coli* phosphatidylethanolamine bilayers at 50 °C (A) and 1,2-dipalmitoyl-*sn*-glycero-3-phosphoethanolamine (DPPE) bilayers at 75 °C (B) perdeuterated in the glycerol backbone. Assignments are given on the top along with the structural description.

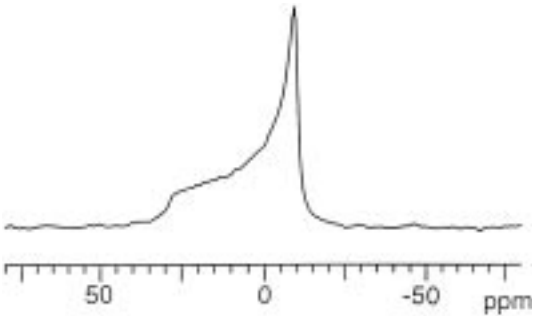

Figure 2. A ^{31}P NMR spectrum of deuterated *E. coli* phosphatidylethanolamine bilayers at 55 °C under proton-decoupling.

either pro-S or pro-R configuration (except for C2). The doublets of the C1R deuteron signals can be seen. Thus, the characteristics reported by Yoshikawa *et al*. have been reproduced even in PE lipid bilayers. The intensity ratio of the doublet, however, changed depending on the culture lot of *E. coli* cells. For example, the C1R signals with a larger quadrupole splitting are weaker than the others in Figure 2A. Actually, the intensity ratio of the doublet was sensitive to the unsaturated fatty acid content, which was monitored by the methine proton intensity of the double bonds of the hydrocarbonchains in a ^1H NMR spectrum of PE. Very weak signals with the quadrupole splitting of about 11 kHz come from the glycerol head group of trace phosphatidyl-glycerol.[10] ^{31}P NMR spectra of the deuterated *E. coli* PE bilayers were measured to examine the Polymorphic state of the sample. A spectrum at 55 °C is presented in Figure 2, which clearly shows that all PE molecules are in the liquidcrystalline lamellar phase. All spectra measured gave the same features.

To investigate the origin of the doublet signals in the ^2H NMR spectrum of phosphatidylethanolamine bilayers, it is critical to use 1,2-diacyl-*sn*-glycero-3-phosphoethanolamine with fully saturated fatty acids. The phospholipid molecule should be stereospecific. It was reported that racemic molecules give rise to a quite different ^2H NMR spectrum.[11] Therefore, we have synthesized 1,2-dimyristoyl- and 1,2-dipalmitoyl-*sn*-glycero-3-phosphoethanolamine (DMPE and DPPE, respectively) specifically deuterated in the glycerol backbone according to the method described under Materials and Methods. Then, ^2H NMR spectra of deuterated DPPE and DMPE bilayers in the liquid-crystalline state were examined. Figure 1B shows a ^2H NMR spectrum of the DPPE bilayers at 75 °C. The quadrupole splittings of about 17.3 and 0 kHz can be ascribed to the 1R and 1S deuterons, respectively. Each deuteron gives rise to only one quadrupole splitting. No doublet was observed in the temperature ranges examined (from 70 to 75 °C for DPPE bilayers and from 50 to 65 °C for DMPE bilayers) for both bilayers. Therefore, it can be concluded that fully saturated PE bilayers take on the homogeneous backbone conformation.

To clarify the origin of each signals of the doublet, a mixture of *E. coli* phosphatidylethanolamine (EPE) and DMPE was examined. The mixture was prepared by mixing deuterated EPE (EPE*) and nondeuterated DMPE, or EPE* and deuterated DMPE (DMPE*) at a one-to-one ratio by weight. ^2H NMR spectra of two mixtures at 55 °C are presented in Figure 3. In order to see the effect of DMPE* accurately, EPE* from the same culture of *E. coli* cells was used for the two preparations. Unfortunately, the outer component of the C1R doublet of this EPE* sample is weak. However, the outer signals can be clearly identified. On addition of DMPE* in stead of DMPE to EPE*, the outer component became stronger. Therefore, this component can be ascribed to the fully saturated PE. The quadrupole splitting of these signals is 17.5 kHz. This is still smaller than the quadrupole splitting (18.9 kHz) of the deuterated DMPE bilayers at 55 °C, suggesting that DMPE does not form separated bilayers (or vesicles). These results indicates that fully saturated PE molecules form such stable domains that give rise to a separate signal. The signal with the smaller quadrupole splitting in Figure 3 should be ascribed to the domains containing saturated and unsaturated fatty acids.

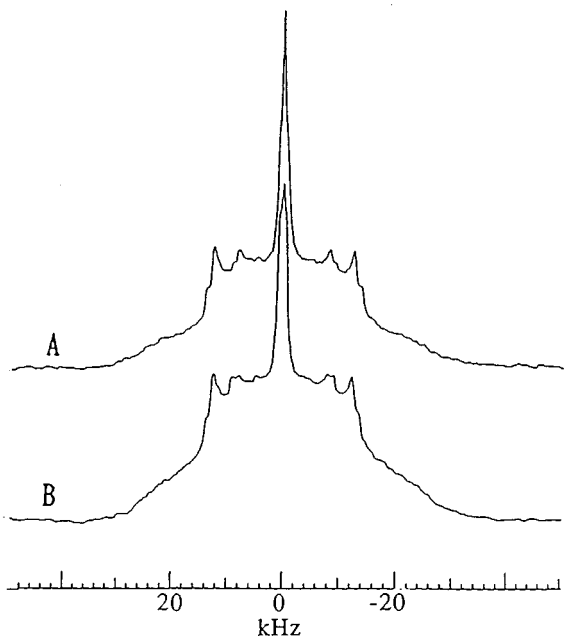

Figure 3. ^2H NMR spectra of the bilayers of the mixture of *E. coli* phosphatidylethanolamine (EPE) and 1,2-dimyristoyl-*sn*-glycero-3-phosphoethanolamine (DMPE) at 55 °C. Mixing ratio is one to one (w/w). (A) deuterated EPE and nondeuterated DMPE. (B) deuterated EPE and deuterated DMPE. The doublets appear at about 8 kHz.

Since no clear doublet can be found for the C3 deuterons in the spectra of Figure 3, it can be concluded that the conformation at the C3 position of the glycerol backbone is the same for different domains. However, the overlapping of signals C2 and C3R makes it difficult to examine if the conformation at the C2 position of the glycerol backbone is the same for different domains. To check the conformation at C2, we have specifically deuterated *E. coli* PE at the C2 position. A ^2H NMR spectrum of its bilayers in the liquid-crystalline state (at 50 °C) is shown in Figure 4. Only one quadrupole splitting can be seen in this spectrum, showing

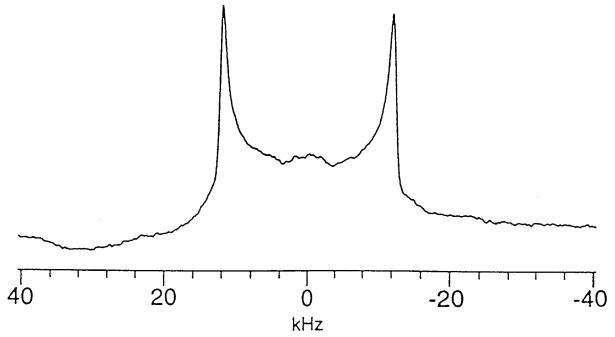

Figure 4. ^{2}H NMR spectrum of *E. coli* phosphatidylethanolamine bilayers at 50 °C specifically deuterated at the C2 position of the glycerol backbone.

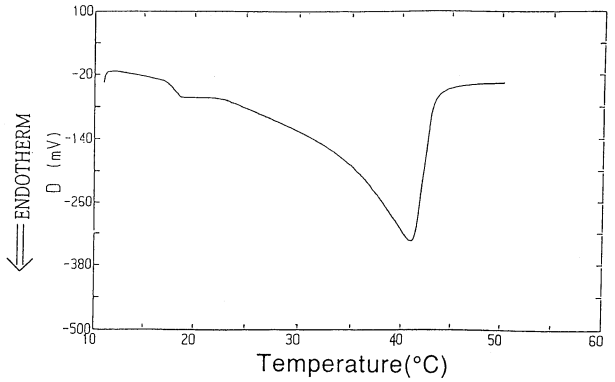

Figure 5. Thermogram of differential scanning calorimetry of (1,2-dimyristoyl-*sn*-goycero-3-phosphoethanolamine + *E. coli* phosphatidylethanolamine) bilayers (1 : 1, w/w).

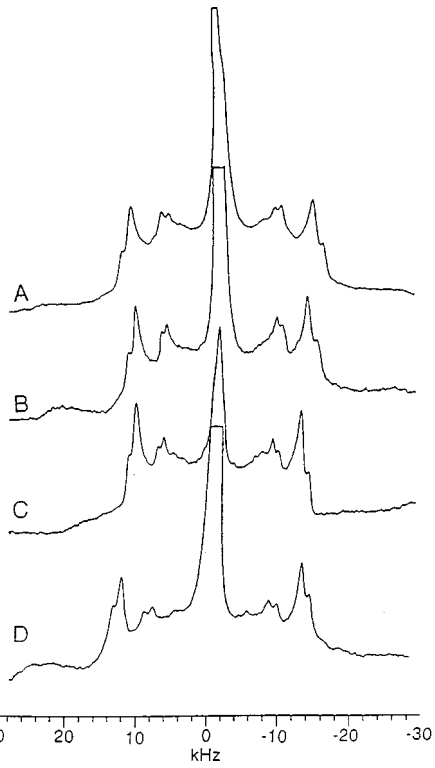

Figure 6. ^{2}H NMR spectra of the deuterated *E. coli* phosphatidylethanolamine (EPE*) mixed with other nondeuterated phospholipids in the liquid-crystalline state (at 50 °C). The glycerol backbone is perdeuterated. Mixing ratio is one to one (w/w). (A) EPE*, (B) EPE* + *E. coli* phosphatidylglycerol, (C) EPE* + *E. coli* cardiolipin, (D) EPE* + 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC).

that the conformation at the C2 position is the same for different domains.

To characterize the bilayers of interest in terms of phase transition, differential scanning calorimetry (DSC) was employed. The DSC thermogram of (DMPE + *E. coli* PE) is presented in Figure 5. This is a typical thermogram for PE bilayers containing heterogeneous molecular species.[12,13,14] There is no separate transition peak at a high temperature expected for the domains with fully saturated fatty acids.

Yoshikawa *et al*. reported the effect of phosphatidylglycerol (PG) and cardiolipin (CL) on the doublet signals.[1] Phosphatidylethanolamine was mixed with one of these acidic phospholipids at a 4-to-1 ratio (w/w). The doublet was still observed in the spectra of those bilayers. Influence of 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC) in addition to *E. coli* PG and *E. coli* CL in the molar ratio of one-to-one (w/w) has been further examined in this work. *E. coli* PE perdeuterated in the glycerol backbone was mixed with non-deuterated other phospholipids. The ^{2}H NMR spectra of these mixed bilayers at 50 °C are presented in Figure 6. As can be seen in the spectra, the doublet was retained in all cases. ^{31}P NMR spectra indicated that only the liquid-crystalline lamellar phase is present for all cases (Spectra are not shown). Therefore, it can be concluded that the two conformations of PE are stable not only in the PE bilayers, but also in the mixed bilayers.

Discussion

When the saturated fatty acid content in *E. coli* membranes is extremely high, each of C1 deuteron of the glycerol backbone of PE gives rise to a doublet, suggesting the presence of two stable backbone conformations. Since the difference in the quadrupole splittings is 1.6 kHz at 50 °C, the exchange rate between two conformations is slower than 10^3 S^{-1}. The ^{31}P NMR spectrum showed that the two conformations of the glycerol backbones are present in the lamellar phase. If the lengths of hydrocarbon chains are very different, mismatch of 1ipid molecules could take place. Such a mismatch can induce a conformational change in the glycerol backbone. The fatty acid composition of *E. coli* PE used in this work was reported as (12 : 0, 1.6%; 14 : 0, 39.7%; 16 : 0 50.7%; 14 : 1, 1.7%; 16 : 1+17Δ, 3.3%; 18 : 1+19Δ, 3.1%),[7] where n : m stands for a fatty acid with n carbons and m unsaturated bonds. NΔ denotes a cyclopropane fatty acid. Since the chain length difference is not significant in the used PE, mismatch of the lipid molecules is unlikely.

Yoshikawa *et al*. suggested two possible reasons for the doublet, namely, a dimer with two different backbone conformations and domains with different fatty acid compositions. ^{2}H NMR spectra of DMPE and DPPE showed that fully saturated PE molecules take on a homogeneous back-

bone conformation in a region of fully saturated PE bilayers, regardless that the region is composed of a dimer or multiple molecules. From the experiments on the mixed bilayers of DMPE and *E. coli* PE (Figure 3), the outer component of the doublet was ascribed to the PE molecules with fully saturated fatty acids. Thus, it can be concluded that there is stable domains of fully saturated PE in the *E. coli* PE bilayers. The thermogram of the mixed lipid indicates that the number of molecules in the domain should be smaller than the lower limit of the cooperative unit of the phase transition. Thus, these domains should be called as microdomains, which do not exclude dimers. Albon and Sturtevant estimated the cooperative unit as 1400 for DPPC bilayers on the basis of calorimetry.[15] Tsong estimated it as 220-250 for DMPC bilayers.[16] Gordeliy et al. proposed the existence of clusters with the average radius of 2.9 nm for DPPC bilayers on the basis of neutron scattering experiment.[17] The cluster would include about 50 molecules. From these values, we can imagine the upper limit of the size of the microdomains of PE bilayers, although we do not know the exact size at this stage.

One might argue that the backbone conformations are intrinsically different for fully saturated PE and unsaturated PE (PE containing unsaturated fatty acids). Namely, a microdomain is just single molecule. If there is an intrinsic backbone conformation, it should be different for different molecular species. Since there are many molecular species in *E. coli* PE in terms of fatty acid composition, the C1 signals should be the overlap of many signals instead of doublets. Thus, it is difficult to assume an intrinsic backbone conformation for single PE molecule. Therefore, we conclude that a microdomain is not just a single molecule.

The conformations of the glycerol backbones are different in the domains with different fatty acid compositions. The conformational difference in the glycerol backbone, however, is restricted to the C1 position. This suggests that the hydrocarbon chain packing determines the backbone conformation. Microdomains with the fully saturated fatty acids would have the highest packing density because of strong inter-polar-head-group interactions of PE including the hydrogen bonding interactions.[6] Such strong interactions can explain the slow exchange rate among the domains.

The microdomains of PE turned out to be so stable that they are retained even in the presence of other phospholipids such as phosphatidylcholine (PC), phosphatidylglycerol (PG) and cardiolipin (CL) at up to 50%. Actually, nonideal mixing in mixed 1ipid bilayers was reported for (DPPC+DPPE) bilayers in the experiments with solid state ^{13}C- and ^2H NMR.[18] Furthermore, it was suggested from calorimetry that PE molecules exhibit a net tendency to self-associate preferentially in the mixed lipid bilayers of PE and PC.[19] They are consistent with the presence of the microdomains of PE molecules. The microdomains can also explains the DSC thermogram pattern in Figure 5. Phase transition starts from the microdomains with unsaturated and/or short fatty acids. Therefore, the thermogram starts to change from very low temperature. Then, at the last stage of the phase transition, only the domains with fully saturated fatty acids are left in the gel state. Thus, the transition takes place in a highly cooperative way, which results in the abrupt termination of the phase transition, making the thermogram asymmetric. Therefore, it is suggested that not only fully saturated PE but also PE with unsaturated fatty acid also form microdomains because of their strong inter-polar-head-group interactions. Since just doublet was observed for C1 deuterons, the molecular exchange among the microdomains should be faster than the NMR time scale ecept for the domains with fully saturated fatty acids.

The asymmetric nature of the phase transition could be used for the protection of bacteria with a high content of saturated fatty acids. On abrupt decrease of environmental temperature, bacteria would be able to survive by keeping a certain part of the membrane in the liquidcrystalline state.[13] The microdomains also could behave as the units in the interaction with membrane proteins and in the transition to the hexagonal II phase. The phase transition of PE from the lamellar to hexagonal II phases is facilitated by the presence of unsaturated fatty acids. Mechanism of this transition have been investigated in terms of the elastic properties of the lipid monolayers and the molecular packing.[20,21] Our results have clearly showed that the incorporation of unsaturated fatty acid induces a conformational change only at the C1 position of the glycerol backbone. However, this does not mean that the conformation of polar head group is not affected. Since the C2-C3 axis is almost parallel to the director axis (bilayer normal),[22] this conformation can accept even a drastic conformational change in the polar head group. Namely, the glycerol backbone can adopt to any changes in the hydrophobic region and in the polar group with the minimum modification of the backbone conformation. Therefore, the glycerol backbone would be the key interface in changing the molecular shape.

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