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Flip-Flop of Phospholipids in DMPC/POPC Mixed Vesicles

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ABSTRACT. Flip-flop rate constants were measured by dithionite assay of NBD-PE fluorescence in DMPC/POPC vesicles made of various DMPC/POPC ratios. The activation energy, enthalpy, entropy, and free energy were determined based on the transition state theory. We found that the activation energy, enthalpy, and entropy increased as the amount of POPC increased, but the activation free energy was almost constant. These experimental results and other similar studies allow us to propose that the POPC molecules included in DMPC vesicles affect the flip-flop motion of NBD-PE in DMPC/POPC vesicles via increasing the packing order of the ground state of the bilayer of the vesicles. The increase in the packing order in the ground state seems to be a result of the effect of the overall molecular shape of POPC with a monounsaturated tail group, rather than the effect of the longer tail group.

Key words: Flip-flop, DMPC/POPC vesicle, Fluorescence

INTRODUCTION

Phospholipid vesicles are important model biomembranes in the diverse biophysical investigations. Their application range is very wide in biological studies such as in sensor development, drug testing, and mechanism elucidation of biological phenomena.[1] Both pure phospholipid vesicles of various sizes and mixed phospholipid vesicles have been used for studying the effects of vesicle size or lipid type on biological phenomena such as peptide-membrane interactions, lipid packing order, and lateral diffusion of lipid molecules.[2–5] Biophysical and biochemical phenomena that occur in/on the biomembranes are deeply related to the structural and dynamic properties of phospholipids forming the biomembrane.[6] Phospholipid molecules have three types of movements in the lipid bilayer.[7] The first type is a rotational motion on its long axis and a wobbling motion; the second type is a lateral diffusion which is a translational motion in two dimensional leaflet; and the third type is a flip-flop motion which is a transbilayer motion from one leaflet to the other leaflet in the bilayer. The lipid flip-flop is an important movement which is involved in many biophysical phenomena such as membrane fun, apoptosis, endocytosis, and asymmetric distribution of lipid species.[8,9]

The mechanism for the flip-flop movement has been investigated at a molecular level using atomic-scale molecular simulation.[9–13] Gurtovenko et al. suggested that the lipid flip-flop takes place in two stages: formation of a water pore, and lateral diffusion and translocation through that pore.[9] Barile et al. found that bending of the alkyl tail of the flipping molecule is the rate-determining step, and thus the chain lengths of both the flipping molecule and the lipid comprising the bilayers affect the flip-flop motion.[13]

The motion is also affected by factors such as proteins (flippase and floppase) in cell membrane, the content of cholesterol, and the kinds of phospholipids. By using coarse-grained molecular dynamics simulations, Ogushi et al. showed that the flip-flop rates of diacylglycerol and ceramide in the 1,2-di-arachidonoyl-sn-glycero-3-phosphocholine (DAPC) membrane correlated with the number of unsaturated bonds in the membrane phospholipids and hence with fluidity of membranes.[14]

Homan et al. reported that the flip-flop rate of pyrenyl phospholipids in POPC vesicle had a smaller dependence on the acyl chain length in contrast to the strong dependence on head group composition.[15] The main reason suggested for the strong dependence on the head group composition was that the insolubility of the polar head group in the membrane interior is the major barrier to flip-flop motion. Inclusion of cholesterol into DOPC vesicles dramatically reduces the flip-flop rate of the lipid due to an increase in either membrane thickness or acyl chain order.[5] The flip-flop rate decreased in vesicles composed of DeuPC with two monounsaturated acyl chains of 22 carbons as compared to those in shorter DPOC or POPC vesicles.[5]

In this study, the effect of addition of 1-palmitoryl-2-oleoyl-glycero-3-phosphocholine (POPC, a common lipid in mammalian membranes) in the 1,2-dimyristoyl-snglycero-3-phosphocholine (DMPC) vesicle on the flip-flop motion

was investigated. POPC molecule has a monounsaturated bond in one of the two tail groups and the length of the tail group is greater than that of DMPC molecule. From the kinetic analysis on fluorescent intensity, the thermodynamic functions of the flip-flop motion of phospholipids in the vesicles with different ratios of DMPC/POPC were elucidated, and the effect of POPC on the flip-flop motion is discussed.

EXPERIMENTAL

Materials

DMPC, POPC, and 1,2-dimyristoyl-sn-glycero-3-phosphoethanolamine-N-(7-nitro-2-1,3-benzoxadiazol-4-yl) (ammonium salt) (NBD-PE) were obtained from Avanti Polar Lipids (Birmingham, AL) and used without further purification. Chloroform and sodium chloride were obtained from Sigma-Aldrich (St. Louis, MO). Tris (hydroxymethyl) aminomethane (Tris) from Bio-Rad (Hercules, CA), Ethylenediaminetetraacetic acid (EDTA) from Samchun (Daejeon, Korea), HCl from Duksan (Gyonggi-do, Korea) and sodium dithionite from Deajung (Gyonggi-do, Korea) were used for preparing a buffer solution.

Preparation of lipid vesicles

DMPC, POPC, and NBD-PE were dispersed in chloroform at the DMPC/POPC molar ratios of 1:0, 6:1, 4:1, and 2:1. The amount of NBD-PE mixed with DMPC/POPC was 1 mol % of unlabeled lipids. The vial containing the lipid mixture was dried in a vacuum desiccator for 8 h. Tris-HCl buffer (NaCl 150 mM, Tris 10 mM, EDTA 1 mM) solution was added to the dried lipid. The final lipid concentration was 2 mM. The multilamellar vesicles were prepared through five freeze-thaw cycles, which consisted of 10 min of vortexing at 30 °C, freezing under liquid nitrogen, and thawing in water bath at 60 °C. Large unilamellar vesicles (LUVs) were prepared through 11 cycles of extrusion, for which polycarbonate filters with 100 nm pore diameter were used.[4]

Dynamic light scattering analysis

Size measurements of LUVs were made using the ELSZ-2000 DLS instrument (Otsuka electronics Co, Ltd, Osaka, JP). Light scattering measurements were performed before and after fluorescence experiments in order to find out the change in vesicle size during the fluorescence measurement.

Fluorescence experiments

To determine the flip-flop rate, labeled LUVs were incubated at temperatures of 25, 30, 35, 40 and 45 °C for 30 min. To find the maximum excitation and emission wavelengths of NBD-PE fluorescence, 'wave scan' experiment was conducted using Scinco FS-2 spectrometer (Daejeon, Korea) and their values were determined to be 470 nm and 530 nm, respectively. Both entrance and exit slit widths were 5 nm. The power of the photo multiplier tube (PMT) was 600 unit. At the best condition for the measurement of the fluorescent intensity an NBD-dithionite assay was performed. The ratio of NBD-PE/dithionite was 1/1000. Dithionite rapidly reduces the fluorescent NBD moiety in the outer leaflet of LUVs to a nonfluorescent moiety. Through the flip-flop motion of NBD-PE the fluorescent intensity of the sample was decreased slowly. From the time profile of the fluorescence intensity the flip-flop rate of NBD-PE molecules could be determined.

Model for the analysis of fluorescence measurement

The flip-flop rate can be determined by measuring the change in fluorescence intensity of NBD-PE when sodium dithionite is added to the vesicle sample.[16] The fluorescence quenching of NBD-PE in lipid vesicles occurs when the NBD-PE molecule is reduced by a dithionite. This reduction occurs only at the outer leaflet because the dithionite is present only on the outer space of vesicles and cannot penetrate the lipid bilayer. The reduction rate of NBD-PE was governed by two reactions shown in Eq. (1): flip-flop motion and quenching reaction.

$$N_I \underset{k_i}{\overset{k_o}{\rightleftharpoons}} N_O \xrightarrow{k_q} N_Q \tag{1}$$

where N_I is the number of NBD-PE molecules in the inner leaflet and N_O is the number of NBD-PE molecules in the outer leaflet. N_Q is the number of the reduced NBD-PE molecules. k_o is the flop rate constant from the inner leaflet to the outer leaflet and k_i is the flip rate constant from the outer leaflet to the inner leaflet. k_q is the quenching (reducing) rate constant of NBD-PE by dithionite. The flip-flop of the reduced NBD-PE molecules need not be considered because the motion does not affect the fluorescent intensity at all. The corresponding differential equation for the number change of NBD-PE molecules is given as below

$$\frac{d}{dt}\begin{pmatrix} N_I \\ N_O \end{pmatrix} = \begin{pmatrix} -k_o & k_i \\ k_o & -k_i - k_q \end{pmatrix}\begin{pmatrix} N_I \\ N_O \end{pmatrix} \tag{2}$$

Under our experimental conditions with high concentration of dithionite (NBD-PE/dithionite = 1/1000), the quenching rate of NBD-PE by dithionite is assumed to be much faster than the flip-flop rate of NBD-PE. We also assume that the flip rate was same to the flop rate to determine the

flip-flop rate.

With these conditions, solving Eq. (2) yields the solution for the fluorescence intensity $I(t)$ at time t after the addition of dithionite:

$$I(t) \propto N_I(t) + N_O(t) = A_1 e^{-k_q t} + A_2 e^{-k_f t} + I(\infty) \quad (3)$$

where $k_f = k_i = k_o$ and $I(\infty)$ is the residual fluorescent intensity.

The flip-flop and the quenching rates are determined by fitting the measured intensity profiles with Eq. (3). The temperature dependence of the flip-flop rates gives the activation energy for the flip-flop of NBD-PE in lipid vesicles through the Arrhenius interpretation.

$$\ln k_f = \ln A - \frac{E_a}{R} \cdot \frac{1}{T} \quad (4)$$

where E_a is the activation energy for the flip-flop of NBD-PE, A is the prefactor of Arrhenius equation, T is the temperature, and R is the gas constant.

Thermodynamic functions for the flip-flop motion can be calculated from the transition state theory and its relation to the Arrhenius activation energy.[15] According to the transition state theory, the reaction rate constant k_f for the flip-flop is as follows

$$k_f = \frac{k_B T}{h} e^{-\Delta G^{\ddagger}/RT} \quad (5)$$

where $\Delta G^{\ddagger}$ is the activation free energy, k_B is the Boltzmann constant, h is the Planck constant.

The activation enthalpy ($\Delta H^{\ddagger}$), the activation entropy ($\Delta S^{\ddagger}$), and the activation free energy ($\Delta G^{\ddagger}$) for the activated state are calculated as follows using the Arrhenius equation.

$$\Delta H^{\ddagger} = E_a - \mathrm{RT} \quad (6a)$$

$$\Delta S^{\ddagger} = \frac{\Delta H^{\ddagger}}{T} + R \ln\left(\frac{h k_f}{k_B T}\right) \quad (6b)$$

$$\Delta G^{\ddagger} = \Delta H^{\ddagger} - T\Delta S^{\ddagger} \quad (6c)$$

RESULTS AND DISCUSSION

As an example, *Fig.* 1 shows the size distribution of DMPC/POPC vesicles before and after dithionite assay. The size distribution shows the standard distribution of LUVs obtained by extrusion process. This means that our extrusion method was adequate for the LUV formation with all the ratios of DMPC/POPC. The average size range at the various DMPC/POPC ratios is 125 ~ 143 nm as shown in *Table* 1. The vesicle sizes were almost same in the exper-

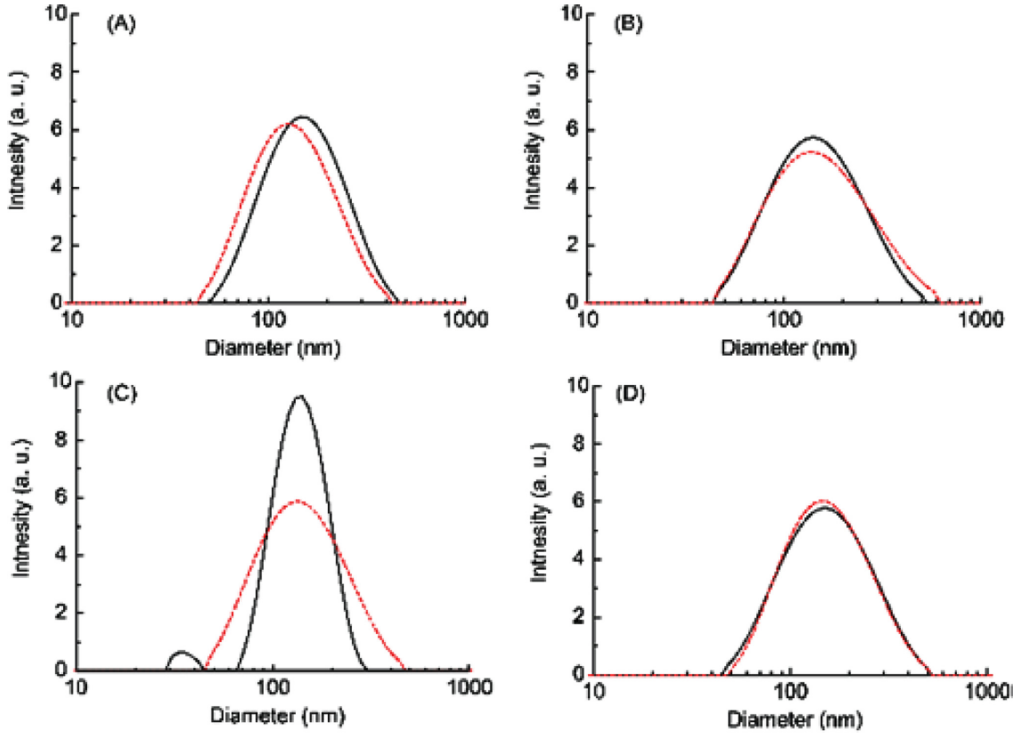

***Figure* 1.** DLS spectra at the DMPC/POPC ratios of 1:0 (A and B) and 2:1 (C and D). (A) and (C) are at 25 °C, and (B) and (D) are at 45 °C. Solid and dashed curves are DLS spectra before and after dithionite assay, respectively.

***Table* 1.** Average size of DMPC vesicles before and after sodium dithionite quenching assay

DMPC/POPC	25 °C		45 °C	
	Before (nm)	After (nm)	Before (nm)	After (nm)
1:0	125.0 ± 0.8	125.0 ± 1.5	130.0 ± 1.4	125.0 ± 2.0
6:1	138.4 ± 10.0	143.0 ± 0.6	136.0 ± 0.4	171.0 ± 86.0
4:1	142.7 ± 8.6	140.2 ± 1.6	132.0 ± 1.2	134.0 ± 1.2
2:1	139.0 ± 1.2	145.0 ± 1.0	134.0 ± 1.8	133.0 ± 0.3

imental temperature range. The vesicle sizes did change a little after dithionite assay, which means that addition of sodium dithionite did not destruct the LUVs during the quenching process. Furthermore, these values did not show any decreasing or increasing trend as the ratio of POPC/DMPC increased. The vesicle sizes in the range of our distribution were known to not affect the flip-flop rate of the lipids.[17] Thus the size effect of vesicle on the flip-flop rate was not considered in our analysis.

Dithionite quenching assay shows fluorescence intensity change with time in *Fig*. 2. In order to determine the exact rate constants for the flip-flop, we performed the dithionite assay in triplicates under the same experimental conditions. All the experimental results are given in supplementary. As expected, the intensity decreasing rate was faster as the reaction temperature increased. The intensity decay was well fitted with the two-exponential decay functions of Eq. (3). The best-fitted curves are shown in the supplementary. The flip-flop rate constants k_f of NBD-PE and diffusion-controlled quenching rate constants k_q of NBD-PE in the outer leaflet were obtained from the best fitting of the intensity change.

The flip-flop rate constants determined from the best fit of fluorescence decay of NBD-PE in DMPC/POPC vesicles in the temperature range of 25~45 °C are given in *Table* 2. Many different values of the lipid flip-flop rate have been reported.[6,18,19] Their differences resulted from the differences in the detection methods and the sample preparations. Sum-frequency vibrational spectroscopy, time-resolved small-angle neutron scattering, and fluorescence spectroscopy were used for the planar supported lipid bilayers, LUVs, and fluorescence-probe molecule-included SUVs or LUVs. For example, the flip-flop rate of DMPC in isotopically asymmetric planar supported lipid bilayers

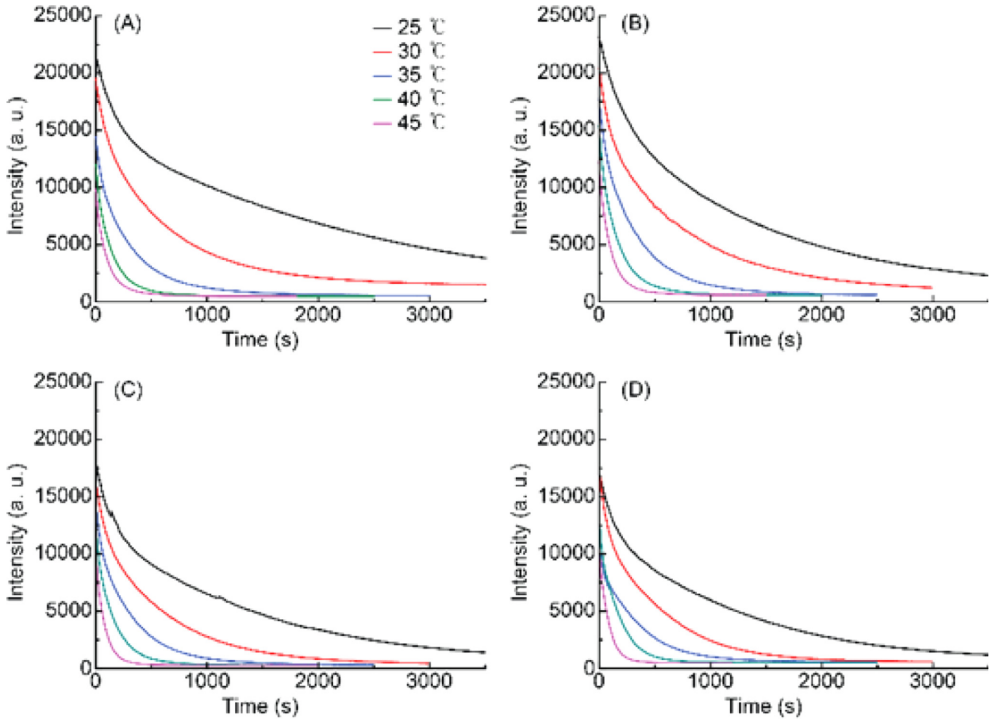

***Figure* 2.** Decay of fluorescence intensity as a function of time at the temperature range of 25~45 °C. The DMPC/POPC ratios are (A) 1:0, (B) 6:1, (C) 4:1, and (D) 2:1.

***Table* 2.** Flip-flop rate constants of NBD-PE in DMPC/POPC vesicles

T (°C)	k_f (s^{-1})×10^3			
	DMPC/POPC			
	1 : 0	6 : 1	4 : 1	2 : 1
25	0.48 ± 0.18	0.60 ± 0.08	0.65 ± 0.03	0.50 ± 0.18
30	1.65 ± 0.25	1.55 ± 0.21	1.23 ± 0.25	1.45 ± 0.30
35	3.05 ± 0.19	2.78 ± 0.30	3.10 ± 0.24	2.48 ± 0.33
40	5.80 ± 0.53	6.23 ± 0.59	5.43 ± 0.33	5.78 ± 0.45
45	6.55 ± 1.78	7.45 ± 0.86	10.60 ± 4.46	11.18 ± 1.13

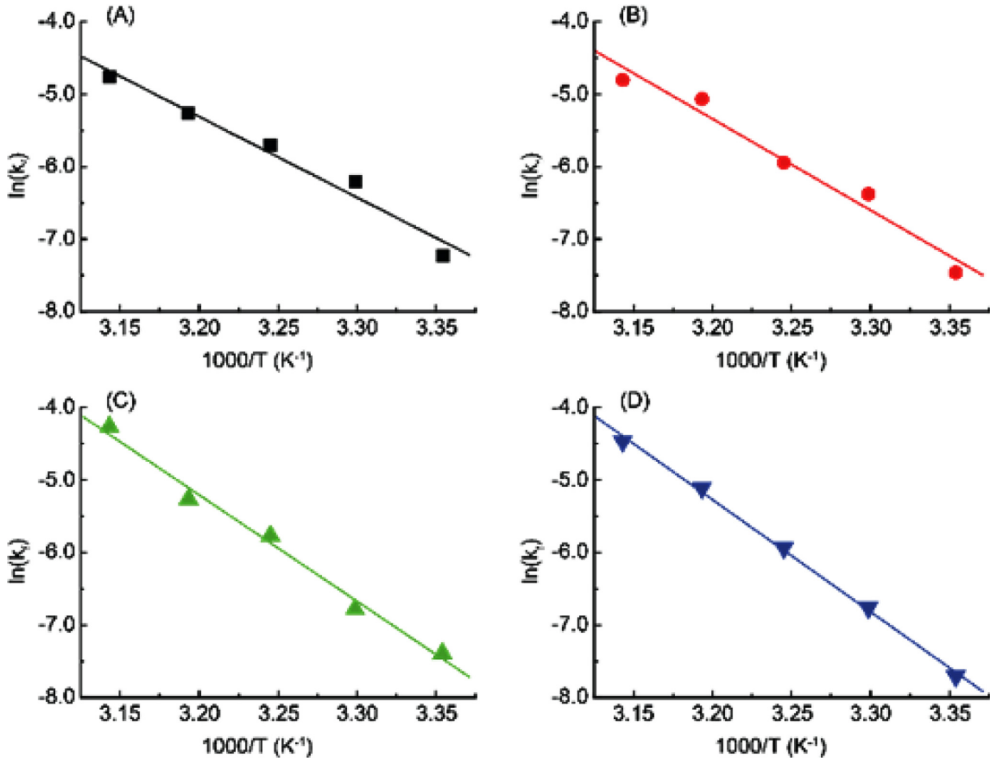

***Figure* 3.** Arrhenius plots of the rate constants for the flip-flop rate of NBD-PE in DMPC/POPC LUVs. The DMPC/POPC ratio is (A) 1:0, (B) 6:1, (C) 4:1, and (D) 2:1.

(PSLBs) was 5.64×10^{-3} s^{-1} at 20 °C.[3] While in our case the flip-flop rate of NBD-PE in DMPC vesicles was $4.8\times10^{-4}s^{-1}$ at 25 °C. The differences in the lipid flip-flop rates between these two experiments might have originated from many causes, one of which could be a defect (submicron-sized holes resulting from incomplete surface coverage)-mediated acceleration of lipid translation in PSLB.[17] Our slower rates in the vesicles without the defect as compared to those of PSLBs were thought to be appropriately measured.

Arrhenius plots of the flip-flop rate constants of NBD-PE are shown in *Fig.* 3. The experimentally determined k_f values show good temperature dependence of Arrhenius type. From the linear-least-squares fit of the data by using Eq. (4), the values of the activation energy E_a for the flip-flop of NBD-PE were determined to be 98 to 126 kJ/mol. Activation energies obtained in this study are very similar to the values (122 kJ/mol) in the previous study.[17] The E_a values were almost linearly dependent of the amount of POPC (*Table* 3).

The activation enthalpy ($\Delta H^{\ddagger}$), the activation entropy ($\Delta S^{\ddagger}$), and the activation free energy ($\Delta G^{\ddagger}$) for the flip-flop motion were calculated by using the Eq. (5) and (6) as shown in *Table* 3. The activation free energy (73 kJ/mol = 17.4 kcal/mol) is very similar to the value determined by the rate constants measurement of saturated FFA with the acyl chain length of the 16 methylene in LUV.[4] As the

***Table* 3.** Activation energy and thermodynamic functions

DMPC/POPC	E_a (kJ/mol)	$\Delta H^{\ddagger}$ (kJ/mol)	$\Delta S^{\ddagger}$ (J/mol· K)	$\Delta G^{\ddagger}$ (kJ/mol)
1 : 0	98 ± 5	96 ± 5	17 ± 16	73 ± 7.1
6 : 1	105 ± 2	102 ± 2	39 ± 7	73 ± 2.8
4 : 1	118 ± 6	115 ± 6	81 ± 20	73 ± 8.5
2 : 1	126 ± 3	123 ± 3	105 ± 10	73 ± 4.3

amount of POPC increased, the activation enthalpies and the activation entropies increased. However, the activation free energies were nearly constant. The increasing enthalpic contribution to the activation free energy for the flip-flop was cancelled by the increasing entropic contribution as the amount of POPC increased.

As observed from the DLS spectrum, the LUVs were well constructed and the same size of LUVs were well maintained in different DMPC/POPC ratios, during the dithionite assay in the experimental temperature range of 25 to 45 °C. Theoretical two-exponential functions fitted the experimental decays of the fluorescence intensity well, and the quenching rates and flip-flop rates in dithionite assay were well determined. The fast quenching rates indicated that the concentration of quenching agent dithionite was high enough to be able to get the slow flip-flop rate constants effectively by fitting the experimental fluorescence decay curves. All these results mean that our theoretical model, with some assumptions and the experimental conditions were very appropriate to determine the flip-flop rate constant of NBD-PE molecules in vesicle solutions.

At first glance, we expected that the activation energies for the lipid flip-flop will decrease via a decreasing acyl chain order of the bilayers in the LUVs due to the inclusion of unsaturated POPC molecules, as the amount of POPC increases.[4]

But the experimental values increased as the amount of POPC increased. The addition of POPC to the DMPC bilayer in the LUVs has two kinds of effect on the lipid flip-flop motion by modifying the DMPC bilayers. One is increase in membrane thickness because the palmitoyl chain of POPC is two carbons longer than the myristoyl chain of DMPC. The other may be the decrease in acyl chain order because of the presence of a monounsaturated carbon in the oleoyl chain of POPC.

Thermodynamic functions and Membrane thickness/ membrane packing

Allhusen et al. observed that the activation enthalpies were very similar for DMPC, DPPC, and DSPC.[3] They suggested that activation enthalpy depends principally on the chemical identity of the lipid head group. This similarity of the activation enthalpies is consistent with the suggestion that the enthalpic barrier to flip-flop is primarily dependent on head group structure of the lipid in membrane bilayer, due to unfavorable interactions between the head group and the bilayer, so these interactions have little or no dependence on bilayer thickness.[6] All these results were obtained in pure phospholipid bilayers prepared on the planar silica supports. Our results measured in the mixed phospholipid vesicles showed that the activation enthalpies increased as the contents of POPC increased. This means that the unfavorable interaction between the head group of NBD-PE and the bilayer core including longer monounsaturated POPCs mixed into DMPCs is higher than that between the head group of NBD-PE and the bilayer core of DMPCs. This may be caused by the tighter packing of the mixed DMPC/POPC phospholipids. This increment resulted the increment of activation enthalpy and activation energy. By combining these two results, we can conclude that the activation enthalpy due to unfavorable interactions depends both on the chemical identity of the lipid head group and on the packing order of the bilayer core.

Anglin et al. found that the activation entropy decreased as the lipid chain length increased.[6] They explained this phenomenon like this: The decreasing trend in the activation entropy with increasing chain length is potentially due to both increasing ground state entropy and/or decreasing transition state entropy. Larger activation entropy for the shorter DMPC as compared longer DSPC is because of a more ordered (tightly packed) ground state of DMPC than those of DSPC, and/or a more ordered transition state of DSPC than that of DMPC. The decrease in activation entropy with increasing chain length is larger than expected based on the changes in the activation area alone, suggesting that the activation entropy is somewhat dependent on membrane thickness as well as lateral packing. In our case, as the content of POPC increased the activation entropy increased. According to the Anglin's explanation, our results mean that the ground state of DMPC/POPC is more ordered (tightly packed) than that of DMPC.

More ordered state of DMPC/POPC than that of DMPC can be explained like this: The increment of bilayer ordering of DMPC/POPC is due to POPC shape. The molecular shape

of POPC is more rectangular than DMPC.[2] The area of head group of DMPC is a little larger than the area of tail group. Insertion of POPC into the DMPC bilayer in the vesicle having a diameter of 200 nm increases the packing order of lipid bilayer and thus decreases the entropy of the ground state. The unsaturated tail group of POPC may increase the disordering of the DMPC/POPC transition state. When the head group of NBD-PE lies in the middle of bilayer in the transition state, it might result in a larger bilayer core space because the unsaturated tail group of POPC moves more freely in the transition state than in ground state. Thus, the entropy of transition state of the DMPC/POPC bilayer increases. This mixing effect of POPCs affect the activation entropy and the activation enthalpy as well.

Anglin et al. observed that increase in activation free energy resulted predominantly from the decrease in activation entropy in the longer phospholipid bilayers.[6] But in our case, the activation free energy is almost same because the activation enthalpy and activation entropy increase by the same amount. The effect of inclusion of POPC in DMPC vesicles on the flip-flop motion is the combination of two opposite effects of the unsaturated tail group and longer tail group of POPC. The thermodynamic effect of the longer chain of POPC on the flip-flop of NBD-PE seemed to bring about no change in activation enthalpy, but decreased the activation entropy; whereas the thermodynamic effect of an unsaturated acyl chain of POPC seemed to increase both the activation enthalpy activation entropy, where the increase in entropy was more than its decrease by the longer chain of POPC.

Barile et al. suggested that flip-flop diffusing proton carriers must bend to minimize the accumulation of stress in the lipid layer.[13] This bending is affected by the alkyl chain length of lipid. Inclusion of cholesterol into the lipid vesicles dramatically decreased the flip-flop rate, which might be from cholesterol's ability to increase the membrane thickness and/or acyl chain order.[5] In order to know how POPC molecules affect the flip-flop motion, further detailed investigation at the molecular level, including the bending of flipping molecule, NBD-PE will be needed. Bennett et al. also found that the transition from initial water defect to pore formation in a DMPC bilayer causes a sudden increase in unfavorable entropy (negative reaction entropy, about -317 J/mol K), and a favorable change in enthalpy of about -50 kJ/mol.[20] These contrasting observations against our results means that the water pore formation in the DMPC/POPC bilayers is not dominant part in the total process of the lipid flip-flop. Sapay et al. observed pore formation in DLPC, DMPC, and DPPC but not in POPC and DOPC.[11] A detailed investigation of mixed DMPC/POPC vesicles at the molecular level vesicles will give a clearer understanding about this phenomenon.

CONCLUSION

The effect of POPC in DMPC/POPC vesicle on the flip-flop motion of NBD-PE molecule was investigated using fluorescence spectroscopy. Increase in the amount of POPC in DMPC/POPC vesicles increased the activation enthalpy and the activation entropy but did not change the activation free energy. The change in thermodynamic values for the flip-flop motion is attributed to the combination of change in unfavorable interaction enthalpy between the head group of flipping molecule and the bilayer core, and the entropy change from the ground state to the transition state during the flip-flop process. These results reconfirm the previous suggestions that the chain length and packing order of lipid molecules in the bilayer of the vesicles strongly affect the flip-flop motion of lipid molecules. Our results show that the ordering effect of POPC with an unsaturated tail group is larger than the increasing length effect.

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