Effect of Artificial Boundary Lipid on the Membrane Dynamics of Human Glycophorin-Containing Liposome

Mitsuaki GOTO† and Junzo SUNAMOTO*
Laboratory of Materials Science of Polymers and Artificial Cell Technology,
Department of Polymer Chemistry, Kyoto University,
Sakyo-ku, Yoshida Hommachi, Kyoto 606
†Nagasaki University, Faculty of Engineering, Department of Industrial Chemistry, Nagasaki 852
(Received July 10, 1992)

Membrane dynamics of egg yolk phosphatidylcholine (egg PC) lipid bilayer which contains human glycophorin with an artificial boundary lipid, 1,2-dimyristoylamido-1,2-deoxyphosphatidylcholine (DDPC), were investigated by ESR spin probe technique. 2-Tridecyl-2-(4-hydroxy-4-oxoundecyl)-4,4-dimethyl-3-oxazolidinyloxy (5-NS), 2-octyl-2-(7-ethoxy-7-oxoheptyl)-4,4-dimethyl-3-oxazolidinyloxy (8-ENP), and 2-hexyl-2-(11-methoxy-11-oxoundecyl)-4,4-dimethyl-3-oxazolidinyloxy (12-MNS) were used as the spin probe to measure the fluidity in different regions and depths of the lipid bilayer membrane. Studies on lipid bilayer containing 5-NS revealed that the reconstitution of glycophorin to egg PC bilayer slightly decreased the membrane fluidity in the vicinity of membrane surface. Further addition of DDPC to this liposome brought about further decrease in the fluidity of this region. Liposomes containing 12-MNS revealed, however, that the addition of glycophorin had an opposite effect on the deep hydrophobic domain of the membrane by increasing the fluidity and the addition of DDPC to the liposome makes this domain less fluid. Corresponding changes were observed in the energy of activation for the rotation of 12-MNS during each case.

The interaction between lipids and proteins in membrane has been extensively investigated since Jost et al.[1] first proposed the existence of boundary lipid around membranous cytochrome oxidase using ESR spin probe technique. It also has been reported[2] that most of membrane-bound enzymes loose their activity if the boundary lipid such as sphingomyelin[3] was lost from the membrane. In this sense, the boundary lipid is considered essential for localization, stability, and the function of membrane proteins and enzymes.

As glycophorin is a major intrinsic glycoprotein of erythrocyte membranes of animals,[4-8] many investigations of the interaction between glycophorin and lipids have been made by various techniques such as DSC,[9] Raman spectroscopy,[10] ^{13}C NMR,[11,12] ^{1}H NMR,[13] ^{2}H NMR,[14] ^{31}P NMR,[15,16] FT-IR,[17] and ESR,[18] to understand the behavior of the boundary lipid. We also study the incorporation of glycophorin into the liposomes containing artificial boundary lipid.

We have recently synthesized an artificial boundary lipid, 1,2-dimyristoylamido-1,2-deoxyphosphatidylcholine (DDPC),[19] to make a regular lecithin liposome physicochemically[19] and biochemically[20] more stable. The physicochemical properties of liposome containing DDPC were well characterized using fluorescence depolarization, DSC,[19] FT-IR,[21,22] and ^{2}H NMR.[14] As expected, the incorporation or the reconstitution efficiency of glycophorin into DDPC-containing egg PC liposome was much higher than that into sphingomyelin-containing egg PC liposome.[23] This improved reconstitution efficiency was attributable to the formation of a stable hydrogen-belt in the lipid bilayer[14,21,22] between the amide groups of DDPC and glycophorin.

In this study, ESR spin probe technique was employed in order to determine the effect of DDPC on the membrane dynamics of human glycophorin-containing egg PC liposomes. For this purpose, three different spin probes, 5-(4,4-dimethyloxazolidine-*N*-oxyl)stearic acid (5-NS), ethyl 8-(4,4-dimethyloxazolidine-*N*-oxyl)palmitate (8-ENP), and methyl 12-(4,4-dimethyloxazolidine-*N*-oxyl)stearate (12-MNS) were employed as the spin probe to measure the fluidity in different regions (or depths) of the lipid bilayer membrane.

Materials and Methods

Materials. Egg phosphatidylcholine (egg PC) was isolated from fresh egg yolk and purified according to the method already established.[23] 1,2-Dimyristoylamido-1,2-deoxyphosphatidylcholine (DDPC) was the same that synthesized and used in previous works.[14,20-28] (DDPC is now commercially available from Dojindo Chemicals, Kumamoto). 2-Tridecyl-2-(4-hydroxy-4-oxoundecyl)-4,4-dimethyl-3-oxazolidinyloxy (5-NS), 2-octyl-2-(7-ethoxy-7-oxoheptyl)-4,4-dimethyl-3-oxazolidinyloxy (8-ENP), and 2-hexyl-2-(11-methoxy-11-oxoundecyl)-4,4-dimethyl-3-oxazolidinyloxy (12-MNS) were purchased from Sigma and used without further purification. Human glycophorin was exactly the same as that used in previous works.[14,20,23]

Liposome Preparation. Egg PC (14.0 mg) or a mixture of egg PC (11.0 mg) and DDPC (2.5 mg, equivalent to 20 mol%) was dissolved in 4.0 ml of chloroform–methanol (3 : 1, by vol) in the presence of a spin probe (0.07 mg) and then 0.4 ml of water or the same volume of an aqueous glycophorin solution was added in a round-bottom flask. The resulting mixture was evaporated in vacuo to obtain a lipid thin film at the bottom of the flask. The glycophorin concentration was kept constant at 0.01 or 0.03 mol% of the total lipids. The lipid

† Present address: Kanagawa Academy of Science and Technology, KSP 3-2-1 Sakado, Takatsu-ku, Kawasaki-shi, Kanagawa 213.

thin film so obtained was dispersed in 2.0 ml of an aqueous buffered solution (20 mM Tris-HCl containing 200 mM NaCl) on a Vortex mixer for 20 min to give multilamellar vesicles (MLVs) and then sonicated at 4.0 °C and 40 W for 10 min under nitrogen atmosphere using a probe-type sonifier (Tomy, UR-200).

ESR Measurements. Electron spin resonance (ESR) measurements were made on a JEOL JES-FX1XG equipped with a thermo-control unit, JEOL ES-DVT using a flat cell of 1.0 mm thickness (JEOL ES-LC11): modulating frequency, 9.236 Hz; magnetic field strength, 3300±100 G; modulation, 100 Hz (2 G); and sensitivity, 0.3.

For a spin probe that hardly undergoes isotropic rotation but anisotropically rotates only around z-axis (for example, spin probe 5-NS) (see Fig. 1-a), we are able to calculate the order parameter (S) using Eq. 1:

$$S=1/2(3\cos^2\gamma-1)=(A_{//}-A_{\perp})/[A_{zz}-1/2(A_{xx}+A_{yy})] \quad (1)$$

A-Tensors ($A_{//}$ and $A_{\perp}$) and hyperfine coupling constant ($a_{N'}$) are obtainable directly from spectrum and Eq. 2:

$$a_{N'}=1/3(A_{//}+A_{\perp}) \quad (2)$$

Because the value of isotropic hyperfine coupling constant (hfc) of the radical (a_N) is affected by being placed in the lipid bilayer membrane, however, if the physicochemical property of the membrane changes, A-tensors also change. Therefore, the corrected order parameter of the spin probe (S') is obtained by Eqs. 2—4:

$$a_N=1/3(A_{xx}+A_{yy}+A_{zz}) \quad (3)$$

$$S'=\frac{A_{//}-A_{\perp}}{A_{zz}-1/2(A_{xx}+A_{yy})}\frac{a_N}{a_{N'}} \quad (4)$$

where the intrinsic A-tensors for spin labelled fatty acids are the following; A_{zz}=33.6, A_{xx}=6.3, and A_{yy}=5.8.[29]

On the other hand, for a spin probe in which takes place relatively rapidly with less anisotropic rotation and gives a sharper triplet in a lipid bilayer compared with the probe described above (see Figs. 1-b and -c), the following relationship is valid for calculating the rotational correlation time ($\tau_c^{(m^2)}$):

$$\tau_c^{(m^2)}=4\left\{\frac{R_{(-1)}+R_{(+1)}-2}{A^2}\right\}\delta H_{(0)} \quad (5)$$

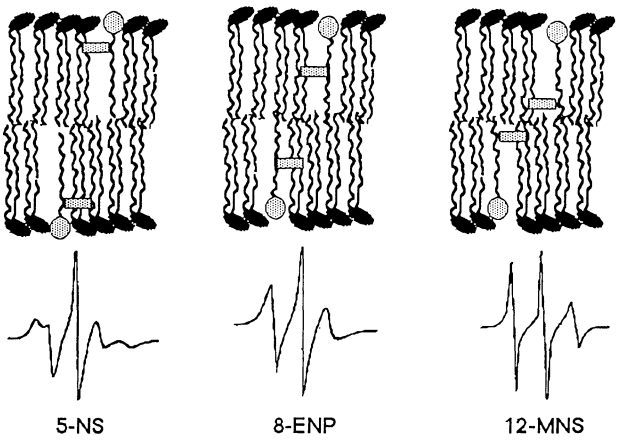

Fig. 1. Expected location of three different spin probes and their spectra simulated.

This is the case for 8-ENP and 12-MNS. If A-tensor of di-t-butylnitroxide radical[30] was employed, data obtained can be analyzed by following equations:[31]

$$\tau_c^{(m^2)}=7.1\times10^{-10}\,\delta H_{(0)}\{R_{(-1)}+R_{(+1)}-2\} \quad (6)$$

$$R_{(m)}=\delta H_{(m)}/\delta H_{(0)}=\{I_{(0)}/I_{(m)}\}^{1/2} \quad (7)$$

$$m=+1, 0, -1$$

(nuclear spin quantum number of nitrogen atom)

where $\delta H_{(m)}$ and $I_{(m)}$ stand for line widths and intensities of the triplet absorption of the nitroxide radical at the lowest (m=+1), the central (m=0), and the highest magnetic fields (m=−1), respectivly.

Results and Discussion

McConnell and his co-workers[32] have first and Nakamura and Ohnishi[33] have then proposed the ESR spin probe method for investigation of dynamics of lipid membranes. When membrane protein is reconstituted into spin-labelled liposome, the ESR spectrum may be affected due to changes in the mobility of lipids around the spin probe. Using this principle, for example, Jost et al. found two kinds of lipid with different mobilities in vesicles and proposed the existence of boundary lipid.[1] Watts and co-worker also investigated the interaction between rhodopsin and membrane lipids in bovine rod outer segment disc membranes.[34] Based on detailed analysis of their data, they also observed two different kinds of lipids; namely, the relatively mobile lipids and the less mobile lipids in direct interaction with rhodopsin.[34] Using the same technique, Utsumi studied the effect of glycophorins of various animals in egg PC liposomal membrane.[18]

If DDPC provides a hydrogen-bonding bridge between matrix lipids and glycophorin reconstituted in the lipid bilayer membrane, the membrane fluidity will be somewhat affected.[11,18] For DDPC-containing DMPC liposomal membranes, we have already reported the increased p-value in fluorescence polarization[19] and increased quadrupole splitting in ^{2}H NMR[14] upon the reconstitution of human glycophorin. In all the previous studies, DDPC was found to always cause a decrease in the fluidity at, especially, the region close to the membrane surface both below and above the phase transition temperature of the membrane. In addition, after the reconstitution of glycophorin into the DMPC liposome, the fluidity of both the membrane surface and the hydrophobic domain rather increased at the temperature below and above the phase transition irrespective of the absence or presence of DDPC.[14,19]

In this work, we employed naturally occurring lipid, egg PC, instead of totally synthesized lipid such as DMPC used in our previous works,[14,19] because the naturally occurring lipid should be more relevant for glycophorin to interact with. In the present work, in addition, three different spin probes, 5-NS, 8-ENP, and 12-MNS, were employed in order to obtain information about different regions and depths of the lipid bilayer

Table 1. ESR Parameters of Glycophorin-Reconstituted Egg PC Liposomes with or without DDPC at 25.0 °C

Probe	Region monitored	Parameter	Liposome[a]			
			I	II	III	IV
5-NS	Close to surface	S'	0.614	0.622 0.613[b]	0.623	0.640 0.619[b]
8-ENP	Midpoint between 5-NS and 12-MNS	$\tau_c^{(m^2)}$/ms	3.19	3.29	3.29	3.37
12-MNS	Deep hydrophobic domain	$\tau_c^{(m^2)}$/ms	1.96	1.83	2.07	2.13

a) Molar ratio of DDPC to egg PC was 20 mol% in all the runs. [Total lipids]=9.2×10^{-3} M, [Glycophorin]=2.8×10^{-5} M (0.3 mol% equivalent to the total lipids), and [Spin probe] =9.2×10^{-5} M (1 M=1 mol dm^{-3}). Liposome I, egg PC; II, egg PC+glycophorin; III, egg PC+DDPC; and IV, egg PC+DDPC+Glycophorin. b) Glycophorin of 0.1 mol% of the total lipids was reconstituted.

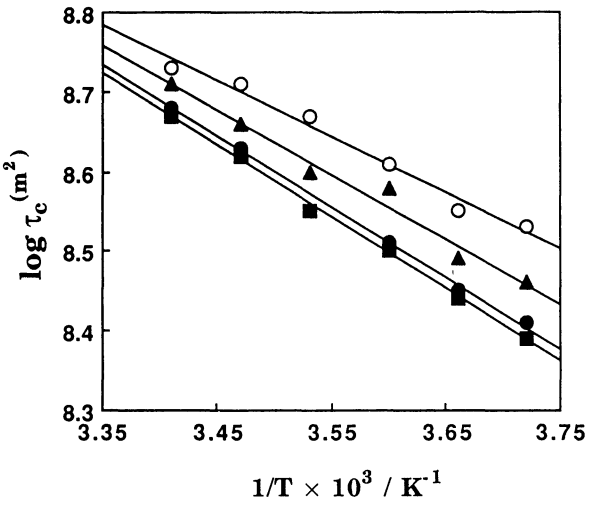

Fig. 2. Temperature dependence of rotational correlation time of 12-MNS in membranes of Liposomes I (▲), II (○), III (●), and IV (■).

Table 2. Energy of Activation of Isotropic Rotation of 12-MNS in Liposomal Membranes

Liposome[a]	I	II	III	IV
ΔE/kcal mol^{-1} [b]	3.7	3.2	4.2	4.1

a) Liposomes I—IV are exactly the same as those given in Table 1. b) Energy of activation was calculated from data determined between −4.0 and +20.0 °C in all the runs.

membrane: Namely, 5-NS gives information about less hydrophobic region close to the surface, 12-MNS gives information about relatively deep hydrophobic domain, and 8-ENP can monitor the midpoint between the regions as informed by 5-NS and 12-MNS. When 5-NS is employed, the order parameter is calculated from the ESR spectrum,[35,36] while the rotational correlation time is obtained for 8-ENP and 12-MNS. Figure 1 shows the location of each probes in a lipid bilayer and their spectra simulated. The order parameter and the rotational correlation time are related to the mobility of the probe and to the relative membrane fluidity. The larger S' and the longer $\tau_c^{(m^2)}$ indicate less membrane fluidity.

In the region close to the membrane surface as monitored by 5-NS, the reconstitution of human glycophorin (0.3 mol%) to egg PC liposomal membrane caused a slight increase in the order parameter (S') from 0.614 of Liposome I to 0.622 of Liposome II (Table I), indicating a decrease in the mobility of lipids in this region. When less amount of glycophorin (0.1 mol%) were reconstituted to the same membrane, however, the mobility of lipids and the membrane fluidity increased rather than decreased. This means that DDPC is more predominant for control of the membrane fluidity. In all the cases, the addition of DDPC more decreased the membrane fluidity at the less hydrophobic region closer to the surface. These findings were in good agreement with those obtained in the fluorescence polarization[19] and ^{2}H NMR[14] studies. Of course, DDPC affects even at the midpoint of the half leaflet of lipid bilayer membrane. Figure 2 shows Arrhenius plots of the rotational correlation time obtained with 12-MNS for Liposomes I—IV, and Table 2 gives their energy of activation calculated from Fig. 2. Clearly, the sequence of the actual fluidity of the deep hydrophobic region certainly decreases in the sequence of egg PC+glycophorin (Liposome II)>egg PC (Liposome I)>egg PC+DDPC (Liposome III)>egg PC+DDPC+glycophorin (Liposome IV) over the temperature range studied. This was more obvious at the lower temperature. However, the order of the energy of activation calculated over the same temperature range was a little bit different from the above sequence; namely, Liposome II < Liposome I ≪ Liposome IV = Liposome III.

Utsumi have investigated[18] the effect of reconstitution of glycophorins of various animals (0.2 or 0.4 mol%) into egg PC liposomal membrane using exactly the same spin probe, 5-NS, as that employed in this work. He observed that though the reconstitution of glycophorin into the egg PC liposome increases the membrane fluidity,[18] however the phenomena is inconsistent with that of ^{13}C NMR studies.[11] Regarding the phenomena at the deep hydrophobic domain of the membrane, our present results were consistent with his ESR studies.[18] On the other hand, regarding the region close to the membrane surface, however, our results agreed with those of ^{13}C NMR studies.[11] In any event, these investigations give us a warning that, whenever the probe method is utilized in the study of membrane dynamics, the sort of probe used, the information available, and the location of the probe in the lipid membrane as well as the limitation of the time scale of the observation, must be carefully considered.

Judging from the results of all the previous and present works such as fluorescence depolarization,[19] DSC,[19] FT-IR,[21] ^{2}H NMR,[14] surface pressure of the lipid monolayer,[22] and the present ESR, we could conclude that our artificial boundary lipid, DDPC, certainly plays an important role for making lecithin bilayer membrane more stable by noncovalent interaction.

References

1) P. C. Jost, O. H. Griffith, R. A. Capaldi, and G. Vanderkooi, *Proc. Natl. Acad. Sci. U.S.A.*, **70**, 480 (1973).

2) H. Sanderman, Jr., *Biochim. Biophys. Acta,* **515**, 209 (1978).

3) C. F. Schmidt, Y. Barenholz, and T. E. Thompson, *Biochemistry,* **16**, 2649 (1977).

4) M. Tomita and V. T. Marchesi, *Proc. Natl. Acad. Sci. U.S.A.*, **72**, 2964 (1975).

5) H. Yoshima, H. Furthmayr, and A. Kobata, *J. Biol. Chem.*, **255**, 9713 (1980).

6) T. Irimura, T. Tsuji, S. Tagami, K. Yamamoto, and T. Osawa, *Biochemisiry,* **20**, 560 (1981).

7) H. Furthmayr, R. E. Garardy, M. Tomita, and V. T. Marchesi, *Arch. Biochem. Biophys.*, **185**, 21 (1987).

8) P. Argos, J. K. M. Rao, and P. A. Hargrave, *Eur. J. Biochem.*, **128**, 565 (1982).

9) E. J. J. van Zoelen, P. W. M. van Dijick, B. De Kruijff, A. J. Verkleij, and L. L. M. van Deenen, *Biochim. Biophys. Acta,* **514**, 9 (1978).

10) T. Taraschi and R. Mendelsohn, *Proc. Natl. Acad. Sci. U.S.A.*, **77**, 2362 (1980).

11) H. Utsumi, B. D. Tunggal, and W. Stoffel, *Biochemistry,* **19**, 2385 (1980).

12) R. L. Ong and J. H. Prestegard, *Biochim. Biophys. Acta,* **692**, 252 (1982).

13) R. L. Ong, V. T. Marchsei, and J. H. Prestegard, *Biochemistry,* **20**, 4283 (1981).

14) J. Sunamoto, K. Nagai, M. Goto, and B. Lindman, *Biochim. Biophys. Acta,* **1024**, 220 (1990).

15) P. L. Yeagle and A. Y. Romans, *Biophys. J.,* **33**, 243 (1981).

16) P. L. Yeagle and D. Kelsey, *Biochemistry,* **28**, 2210 (1989).

17) R. A. Dluhy, R. Mendelsohn, H. L. Casal, and H. H. Mantsch, *Biochemistry,* **22**, 1170 (1983).

18) H. Utsumi, *Adv. Pharm. Sci. (Tokyo)*, **2**, 93 (1986).

19) J. Sunamoto, M. Goto, K. Iwamoto, H. Kondo, and T. Sato, *Biochim. Biophys. Acta,* **1024**, 209 (1990).

20) J. Sunamoto, M. Goto, and K. Akiyoshi, *Chem. Lett.,* **1990**, 2141.

21) T. Kawai, J. Umemura, T. Takenaka, M. Goto, and J. Sunamoto, *Langmuir,* **4**, 449 (1988).

22) D. W. Grainger, J. Sunamoto, K. Akiyoshi, and K. Knutson, *Langmuir,* (1992), in press.

23) J. Sunamoto, M. Goto, M. Arakawa, T. Sato, H. Kondo, and D. Tsuru, *Nippon Kagaku Kaishi*, **1987**, 569.

24) M. Goto, M. Arakawa, T. Sato, H. Kondo, and J. Sunamoto, *Chem. Lett.,* **1987**, 1935.

25) J. Sunamoto, K. Sakai, T. Sato, and H. Kondo, *Chem. Lett.,* **1988**, 1781.

26) J. Sunamoto, M. Goto, and K. Akiyoshi, *Chem. Lett.,* **1990**, 1249.

27) J. Sunamoto, T. Noguchi, T. Sato, K. Akiyoshi, R. Shibata, E. Nakayama, and H. Shiku, *J. Controlled Release,* **20**, 143 (1992).

28) J. Sunamoto, Y. Mori, and T. Sato, *Proc. Jpn. Acad., Ser. B,* **68**, 69 (1991).

29) M. Esmann, A. Watts, and D. Marsh, *Biochemistry,* **24**, 1386 (1985).

30) L. J. Libertine and O. H. Griffith, *J. Chem. Phys.,* **53**, 1359 (1970).

31) K. Ishizu, "Zitsuyou ESR Nyumon," Kodansha Scientific, Tokyo (1981), pp. 145—156.

32) W. L. Hubell, J. C. Metcalfe, S. M. Metcalfe, and H. M. McConnell, *Biochim. Biophys. Acta,* **219**, 415 (1970).

33) M. Nakamura and S. Ohnishi, *Biochem. Biophys. Res. Commun.,* **46**, 926 (1972).

34) A. Watts, J. Davoust, D. Marsh, and P. F. Devaux, *Biochim. Biophys. Acta,* **643**, 673 (1981).

35) W. L. Hubbell and H. M. McConnell, *J. Am. Chem. Soc.,* **93**, 314 (1971).

36) L. M. Gordon and R. D. Sanerheber, *Biochim. Biophys. Acta,* **460**, 34 (1977).