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Effect of the Phase Transition on the Photochemical Reactions in Lipid Bilayer Membranes†

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The effect of phase transition on the photochemical reactions in liposome system containing amphiphilic dye is studied. The fluorescence intensity of the amphiphilic cyanine dye with two long alkyl chains, *N,N'*-distearylthiacarbocyanine bromide, which is incorporated into bilayer liposome, is considerably modified by the fluid–solid phase transition of the lipid bilayer, while the fluorescence of the cyanine dye with one long alkyl chain, *N*-stearyl-*N'*-methylthiacarbocyanine bromide, was not affected by the phase transition. These results were interpreted in terms of the suppression of the twisting motion of the chromophores around the bridging bond in the excited singlet state. Factors affecting the photoinduced coupling of redox reactions across bilayer membrane, i.e. reduction of $Fe(CN)_6^{3-}$ in outside aqueous phase of liposome and oxidation of potassium ascorbate in inside solution, are also studied using chlorophyll *a* incorporating liposome. It is found that phase transition exerts the influence on the fluorescence intensity of chlorophyll *a*, and on the rate of bimolecular reaction of chlorophyll *a* with water-soluble species.

INTRODUCTION

All current models of biological membrane employ the basic concept of smectic phase lipid bilayer.[1] The fluidity of the hydrocarbon chain in a lipid bilayer membrane has been known to play an important role in the functions of the biological membrane. For example, it has been demonstrated that photosynthetic parameters in blue-green alga *Anacystic Nidulans* depend on the fluid–solid phase transition of lipids of membrane flagments.[2] Because of the impor-

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tance of lipid phase transition as stated above, many physicochemical studies have been reported on artificial bilayer membranes, such as liposome, from the viewpoint of relation between physical phase of lipids and membrane functions.[3,4]

Amphiphilic dyes with a hydrophilic chromophore and one or two lipophilic long hydrocarbon chains can be incorporated into liposomal membrane framework giving various functions to the membrane. We have reported that photoinduced redox reactions at the both membrane-solution interfaces of a liposome are coupled across bilayer membrane containing chlorophyll *a*,[5] and that the aggregation of amphiphilic dye incorporated into liposome membrane depends on physical state of hydrocarbon chains of constituent lipids.[6]

In this study, the effect of phase transition of lipids on the photochemical reactions of amphiphilic dye incorporated into liposome system was investigated. First, the intramolecular photochemical process, or intramolecular relaxation of excited state, of incorporated cyanine dye which has one or two long alkyl chains was examined. Next, influence of phase transition on the coupling of photoinduced redox reactions through liposomal membrane containing chlorophyll *a* was studied.

EXPERIMENTAL

Dimyristoylphosphatidylcholine (DMPC) and Dipalmitoylphosphatidylcholine (DPPC) purchased from Sigma Chem. Co., *N,N'*-distearylthiacarbocyanine bromide (DSSCC) and *N*-stearyl-*N'*-methylthiacarbocyanine bromide (MSSCC) from Japanese Research Institute for Photosensitizing Dyes Co. Ltd., and Carbonylcyanide *m*-chlorophenylhydrazone (CCCP) from Aldrich Chem. Co. Inc. were used without further purification. Chlorophyll *a* was extracted from fresh spinach leaves and purified by the method of Iriyama *et al.*[7] The purity of the reagents was checked by thin layer chromatography.

Liposome dispersion was prepared by the following procedure. PC colyophilized with dye from benzene solution under vacuum was suspended in an Ar-saturated aqueous solution of a desired composition. The suspension was ultrasonicated at a temperature by 5° C above the phase transition. The phosphatidylcholine concentration in the liposome dispersion was checked by the Fiscke-Subbarow method.[8] The composition of outside aqueous phase of liposome was exchanged by rapid passage of liposome dispersion on Sephadex G-50 column. Dissolved oxygen was removed by bubbling Ar gas, if necessary.

Absorption measurements were carried out using a spectrophotometer, Beckman 25. Fluorescence measurements were performed on a spectrofluorophotometer, Hitachi MPF2A. The concentration change of $Fe(CN)_6^{3-}$ in the

outer aqueous phase of liposome dispersion was measured during illumination with 500 W Xe lamp through glass filters (490–800 nm) by the use of an ion selective electrode. The bleaching rate of cholorophyll *a* by strong oxdant, such as potassium dichromate, was measured with Union Giken RA-601 stopped flow apparatus. Temperature of sample was thermostated within ±0.1°C by circulating water, and monitored with a thermocouple dipped in the sample cell.

RESULTS AND DISCUSSION

1 Effect of the phase transition on the fluorescence of amphiphilic cyanine dyes

Effect of the membrane fluidity on the intramolecular relaxation process of excited state of amphiphilic cyanine dyes, DSSCC and MSSCC, incorporated in a liposome membrane was investigated by measuring temperature dependence of fluorescence intensity of the cyanine dye in a liposome bilayer dispersed in a buffered solution (0.1M KCl, 0.01M tris-HCl at pH 7.5). The molar ratio of lipid to dye was 4600–7000, thus the average local concentration of the dye was less than 1 molecule/vesicle throughout the experiments.

The fluorescence spectra of the dyes, DSSCC and MSSCC, in liposomes were quite similar to those in methanol solutions. The fluorescence intensity of DSSCC in the liposome were two to six times as large as those of the dye in methanol at 20°C while the fluorescence intensity of MSSCC was twice the intensity of the same dye in methanol. These facts are interpreted qualitatively in terms of the difference of effective viscosity of the media affecting the freedom of motion of the dyes.

The fluorescence intensity of these cyanine dyes in lipsomes was enhanced with decreasing temperature. In Figure 1, logarithm of the fluorescence intensity of DSSCC is plotted against reciprocal of temperature. Figure 1 gives the following temperature dependence of fluorescence intensity,[9] I_t

$$\ln I_t = A + \frac{B}{T} \tag{1}$$

where A and B are constants. Taking into account of phase transition temperature of DMPC or DPPC to be 19.7°C or 36.0°C, the temperature dependence of DSSCC fluorescence intensity may be due to the fluid–solid phase transition of lipids in bilayer membrane. On the other hand, the fluorescence intensity of MSSCC, which has only one long alkyl chains, is little affected by the phase transition as shown in Figure 2. These results were interpreted in terms of the effect of the bilayer membrane fluidity on the rotational motion of the excited singlet state cyanine dyes. It has been reported that twisting mo-

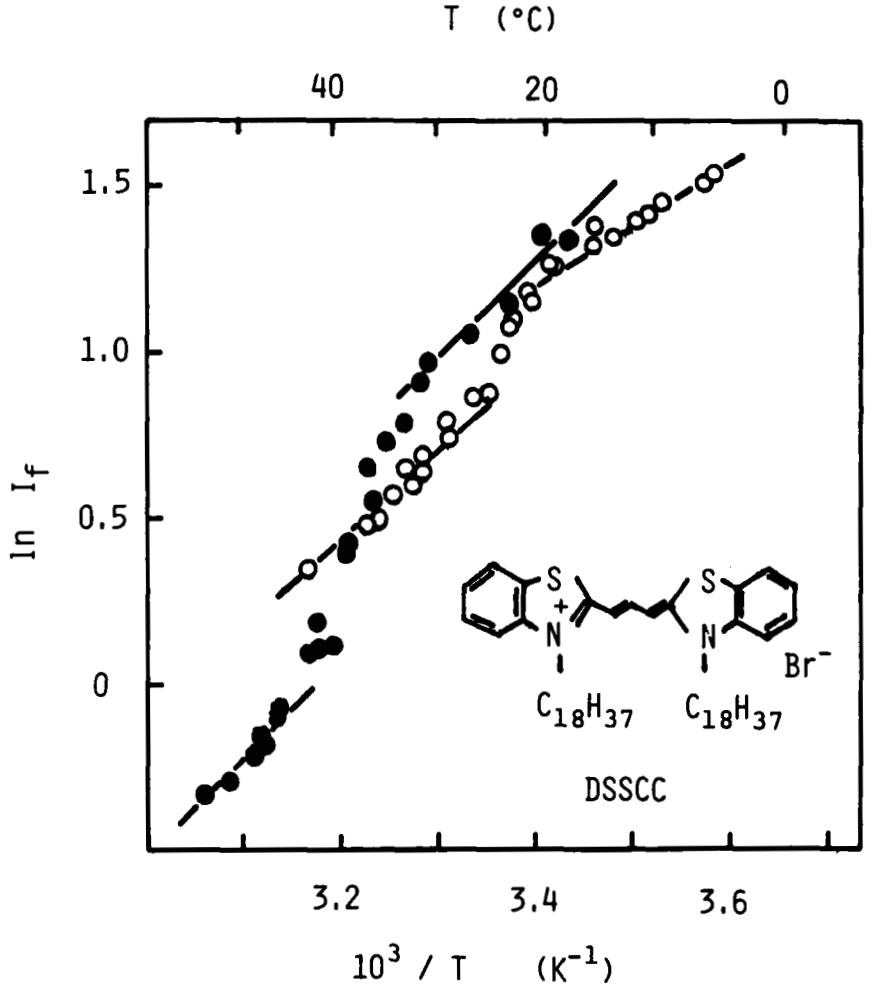

FIGURE 1 Logarithm of the fluorescence intensity of DSSCC *vs.* reciprocal of temperature. (○); DMPC liposomes, (●); DPPC liposomes.

tion of the chromophore around the conjugated hydrocarbon chain play an important role in nonradiative relaxation of excitation energy of thiacarbocyanine dyes.[10] In the case of MSSCC, rotational motion around the bridging bond is not so affected by the membrane fluidity. On the other hand, in the case of DSSCC; whose two long alkyl chains are solubilized deeply into the hydrocarbon region of liposomal membrane, the rotational motion around the bridging bond is much restricted and the freedom of motion is considerably affected by the membrane fluidity modified by the phase transition of the lipid bilayer. Thus, the fluorescence intensity of DSSCC reflects the phase transition of bilayer membrane.

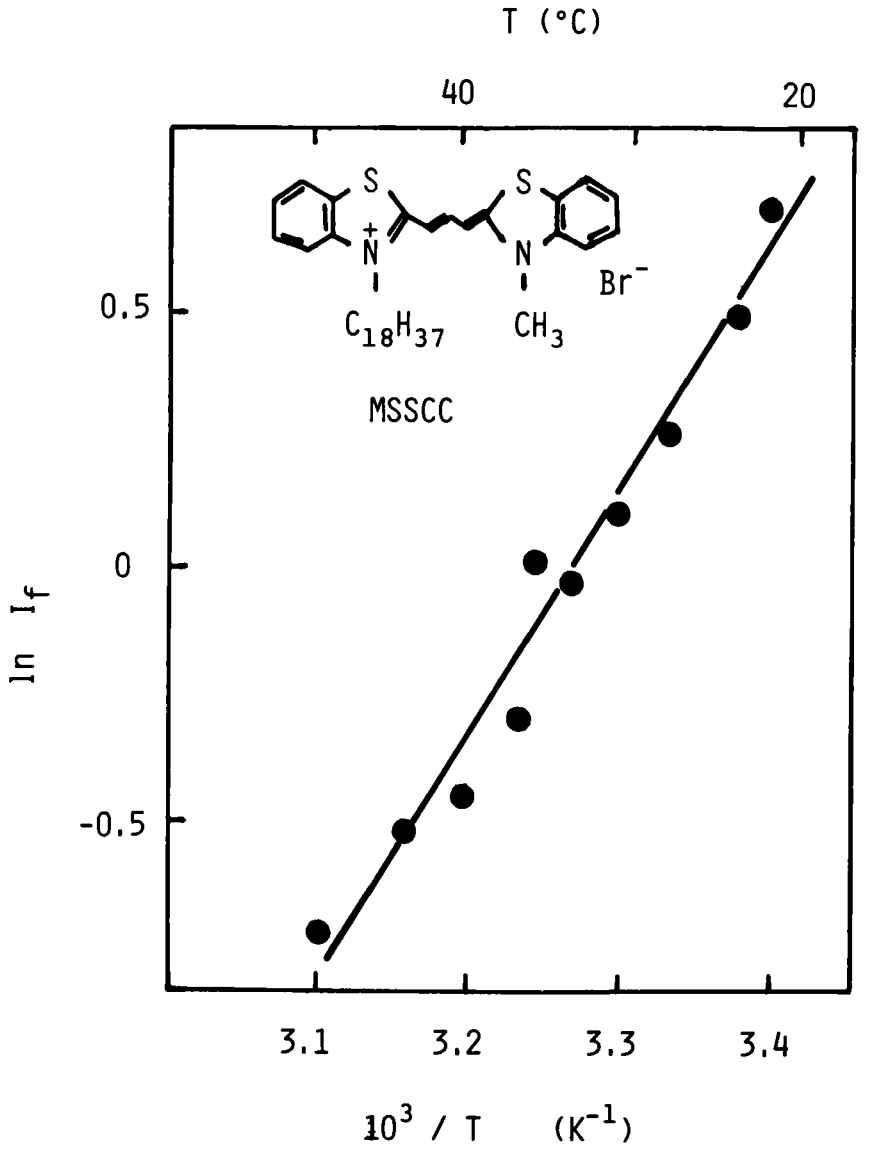

FIGURE 2 Logarithm of the fluorescence intensity of MSSCC in DPPC liposome *vs.* reciprocal of temperature.

The fraction of solid phase region Θ, will be expressed by

$$\Theta = \frac{I_t(T) - I_f(T)}{I_s(T) - I_f(T)} \tag{2}$$

where $I_t(T)$ is the observed fluorescence intensity, $I_f(T)$ and $I_s(T)$ are the

fluorescence intensity of the dye in the fluid phase region and the solid phase region at temperature T, respectively. Figure 3 illustrates temperature dependence of Θ calculated from the DSSCC fluorescence intensity by assuming the same type of temperature dependence for I_l and I_s as Eq. (1) in the whole temperature range.

It is possible to calculate the cluster size of the cooperative transition from the data in Figure 3, if we assume that the partition of DSSCC in the lipid bilayer is not affected by the physical state of the bilayer. The phase transition phenomenon can be regarded as a quasi-first-order reaction with the equilibrium constant, K

$$K = \frac{\Theta}{1 - \Theta} \tag{3}$$

Combination of Eq. (3) with van't Hoff equation leads to the following equation.[11]

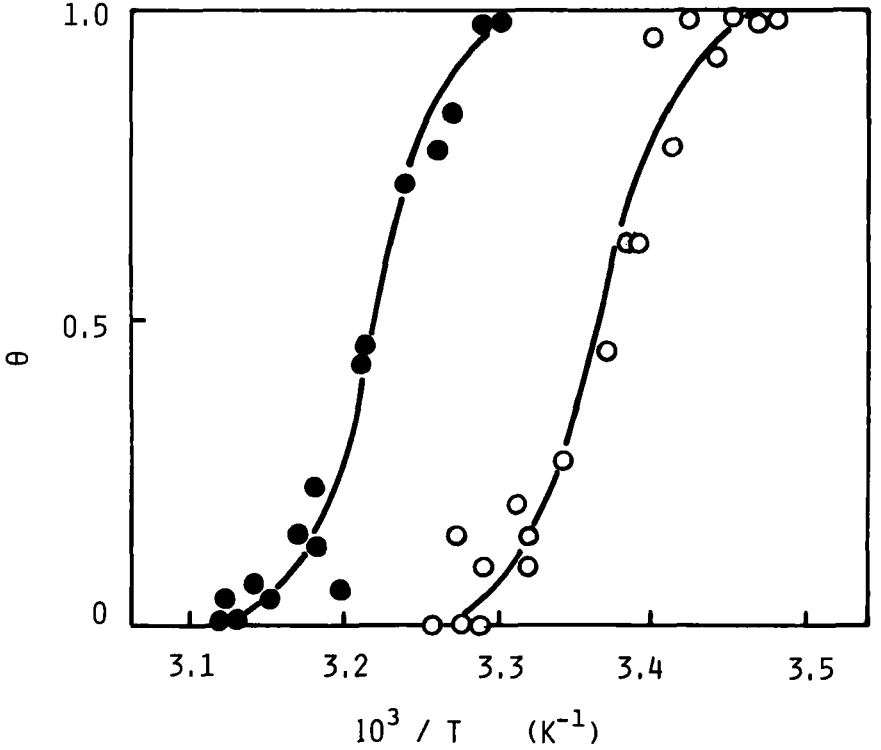

FIGURE 3 Mol fraction of solid phase obtained with the DSSCC fluorescence *vs.* reciprocal of temperature. (○); DMPC liposomes, (●); DPPC liposomes.

$$\left(\frac{d\Theta}{dT}\right)_{T_m} = \frac{\Delta H_{vH}}{4RT_m^2} \tag{4}$$

where T_m is the temperature at the center of the transition and ΔH_{vH} is so-called van't Hoff enthalpy of the transition. Assuming that the calorimetrically obtained enthalpy of transition for multillamellar liposome, ΔH_{cal}, 6.26 kcal/mol for DMPC or 9.69 kcal/mol for DPPC,[12] is also applicable for single bilayer liposome, the cluster size of the cooperative transition ($\Delta H_{vH}/\Delta H_{cal}$) is estimated. The phase transition parameters obtained in the present study are listed in Table I, and those values are well agreed with those obtained by other method.[13]

It is concluded that the fluorescence intensity of the amphiphilic cyanine dyes with two long alkyl chains, such as DSSCC, which are incorporated in bilayer liposomes is considerably affected by the phase transition of the lipid bilayer through the suppression of the twisting motion of the chromophores around the bridging bond in the excited singlet state. DSSCC fluorescence intensity has been found to be very useful tool for examining membrane phase transition, because of its simplicity of use.

2 Effect of the phase transition on the bimolecular redox reactions in liposome system containing chlorophyll *a*

The enhancement of photoreduction of $Fe(CN)_6^{3-}$ in the outer aqueous phase by a reductant such as potassium ascorbate (KAs) localized in a solution of the opposite side of the DMPC membrane containing chlorophyll *a* was observed similarly to that of Cu^{2+}.[4] Here, the compositions of the internal and the external solutions were as follows; internal solution: KAs, 0.1 M KCl, 1.0 M tris-HCl at pH 7.5, external solution: $K_3Fe(CN)_6$, 0.1 M KCl at pH 7.5. This fact suggested that redox reactions at the both membrane-solution interfaces of a liposome were coupled through the bilayer. The addition of CCCP, proton carrier, to the dispersion intensively facilitated the photoreduction. In this case, temperature dependence of photoreduction rate of $Fe(CN)_6^{3-}$ by KAs has not been clarified because of the complexity of the reaction process.

The elucidate influence of phase transition on photoreduction process of $Fe(CN)_6^{3-}$, the effect of phase transition on a bimolecular reaction, bleaching of chlorophyll *a* in DMPC liposome in the dark by a water-soluble strong ox-

TABLE I
Phase transition parameters obtained with the DSSCC fluorescence

Lipid	T_m	ΔH_{vH}	$\Delta H_{vH}/\Delta H_{cal}$
DMPC	23.5°C	68.8 kcal/mol	11
DPPC	37.6	106	11

idant, potassium dichromate, was examined by the stopped flow method. As shown in Figure 4, bleaching rate of chlorophyll *a* was nearly constant above the phase transition temperature and decreased below phase transition. This fact indicates that bimolecular reaction of amphiphilic molecule in liposome bilayer with water-soluble species is affected by membrane fluidity, while it is restricted in the region where lipids are in solid phase. In addition, fluorescence intensity of chlorophyll *a* incorporated into DMPC liposome with or without CCCP was measured by varying temperature. Figure 5 shows that with lowering temperature the fluorescence intensity abruptly decreased accompanying the formation of solid phase. The above results suggest that the phase transition temperature shifted to lower by the addition of CCCP, i.e. lowered by about 5°C at 1.22×10^{-4}M CCCP in 1.0×10^{-3}M DMPC, indicating the decrease of the ordering of lipid hydrocarbon chains in liposome membrane in the presence of lipophilic molecule, such as CCCP. Decrease in

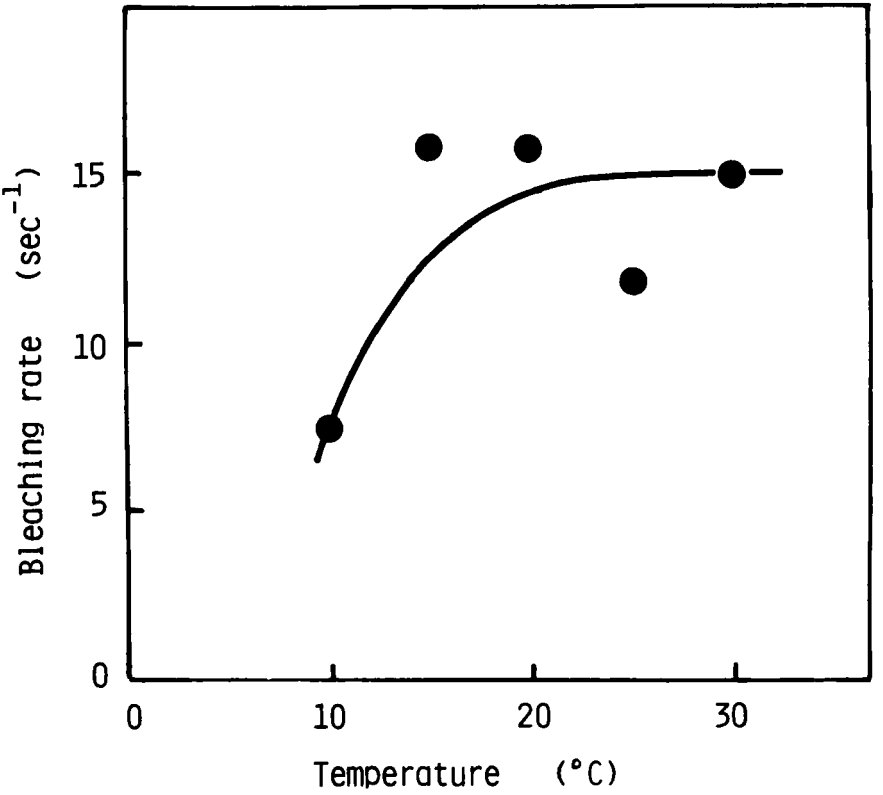

FIGURE 4 Temperature dependence of bleaching rate of chlorophyll *a* in DMPC liposome by 0.17 M potassium dichromate in 0.25 M H_2SO_4. Chlorophyll *a* concentration in liposome dispersion is 1.0×10^{-5} M.

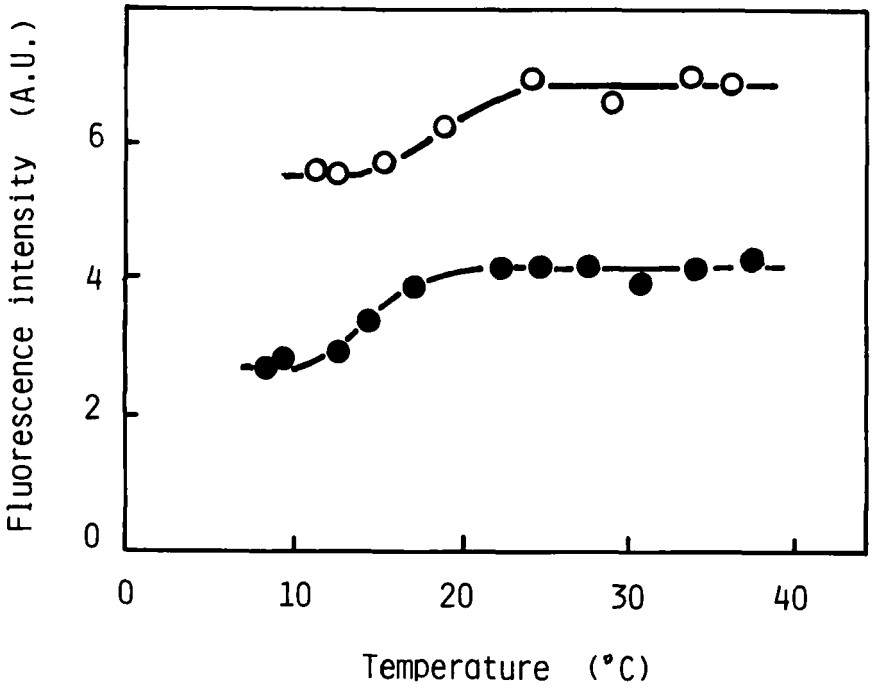

FIGURE 5 Fluorescence intensity of chlorophyll *a* incorporated into DMPC liposome is plotted against temperature. Concentration of DMPC is 1.0×10^{-3} M and the molar ratio of chlorophyll *a* to DMPC is 1/120. (○); without CCCP, (●); with CCCP of 1.22×10^{-4} M.

fluorescence intensity in solid phase may be interpreted in terms of chlorophyll *a* aggregate formation[4] or some other changes of arrangement of chlorophyll *a* excited state in liposome membrane. It is reported that permeation rate of some species in bilayer membrane shows a maximum at the phase transition temperature of lipid,[3,14] because of the existence, of a loosely packed boundary region between fluid and solid phases. Thus, the membrane fluidity is expected to affect the photoreduction rate through the bimolecular reaction rate, organization of chlorophyll *a* or membrane permeability.

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