Fluorescence Studies on the Phase Dependence of Interactions between a Tripeptide, Lys-Trp-Lys, and Dimyristoylphosphatidylserine

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Interactions of a basic tripeptide, lysyl-tryptophyl-lysine (LTL) with dimyristoylphosphatidylserine (DMPS) were investigated by fluorescence spectroscopy. An interacting LTL-DMPS complex assumes two distinctly different conformations in the lipid bilayer depending on the thermotropic phase of lipid. At temperatures below the gel-liquid crystalline phase-transition temperature (T_c), LTL binds to the polar head groups of DMPS through electrostatic force, and the tryptophan residue is located on the surface of the membrane because of the rigidity of the head groups. Above T_c, it assumes a conformation such that the tryptophan residue slips deeply into the DMPS bilayer. The temperature dependence of the fluorescence quenching by acrylamide supported the penetration of the tryptophan upon the phase transition of DMPS. A fluorescence decay analysis based on the photophysical deactivation mechanism of tryptophan residue has revealed a peculiar interaction of LTL with DMPS in the bilayer.

Recently, much attention has been paid to the peptide-lipid interaction because many small peptides, when brought to interact with membrane systems, exert significant biological effects or display important physiological functions. Investigations of their interactions provide information on the relationship between their structures and functions.[1,2] Fluorescence spectroscopy is endowed with some functions which have been proved useful for such studies. Since only tyrosine and tryptophan residues give efficient fluorescence, they provide a specific intrinsic probe for properties of the peptide-lipid system.

A tripeptide, LTL, has attracted a great deal of interest because of its intrinsic fluorescence due to tryptophan and the positively charged lysine residues which are responsible for electrostatic interactions with the negatively charged head group of phospholipid bilayers. There is evidence that upon LTL's binding with phosphatidylserine, the fluorescence emission spectrum shifted to shorter wavelengths accompanying a marked enhancement in quantum yield, and such a change in fluorescence spectrum has been interpreted as being due to a change in the environment of the indole ring of tryptophan.[3] These results directed us to carry out detailed studies on binding properties of LTL with the phospholipid bilayer. The present paper reports of fluorescence spectroscopic studies on interactions of LTL with DMPS above and below the transition temperature of the phospholipid.

Materials and Methods

LTL and 1,6-diphenylhexatriene (DPH) were purchased from Aldrich Chemicals Co. and purified by recrystallization and sublimation, respectively. DMPS obtained from Avanti Polar Lipid Inc. was used without further purification. The other reagents used throughout the present experiment were of analytical grade.

Unilamellar Vesicles of DMPS. Unilamellar vesicles of DMPS were prepared by the method of Barenholz,[4] and the concentration of DMPS was determined by the method of Bartlett.[5]

Fluorescence Spectra. Fluorescence measurements of LTL and its complex with DMPS vesicles were carried out on a Perkin-Elmer spectrofluorometer MPF-44 equipped with a DCSU-2 microprocessor correction unit. Analog signals were passed into an ADC interfaced to a Commodore minicomputer having a Commodore 2040 disk driver for data storage. For spectroscopic analysis of LTL-DMPS interactions, a small aliquot of DMPS vesicles was added to 2 ml of LTL solution in Tris-acetate buffer at a given pH. This was done in such a manner that the concentration of the remaining LTL is kept constant. Unless otherwise specified, fluorescence was measured with excitation at 280 nm and with 3 nm bandpath. Samples were kept at a constant temperature (±0.5 °C) by circulating water through a thermostated bath.

Fluorescence Decay Analysis. Fluorescence decay measurements were performed by use of the technique of time correlated single photon counting. A synchronously pumped dye laser and a cavity dumper system were employed as the excitation source operating at 825 kHz and with 15 ps pulse width. Detection of emissions was performed on a photomultiplier after their successive passage through a polarizer at the magic angle and a monochromater with 4 nm bandpath. The wavelength dependence of the instrument responce was corrected by using an emission from terphenyl. Data were accumulated in 1024 channels of a multichannel analyzer with a channel width of 21.6 ps, and analyzed by the nonlinear least squares iterative deconvolution method based on the Marquardt algorism. The adequacy of exponential decay fitting was examined by inspection of weighted residual plots and other statistical parameters. The details of the instrumentation and the method of data analysis were described in our previous paper.[6]

Results

Fluorescence Spectra of LTL in the Absence and Presence of DMPS Vesicles. Figure 1 shows fluorescence spectra of free LTL and its complex with DMPS

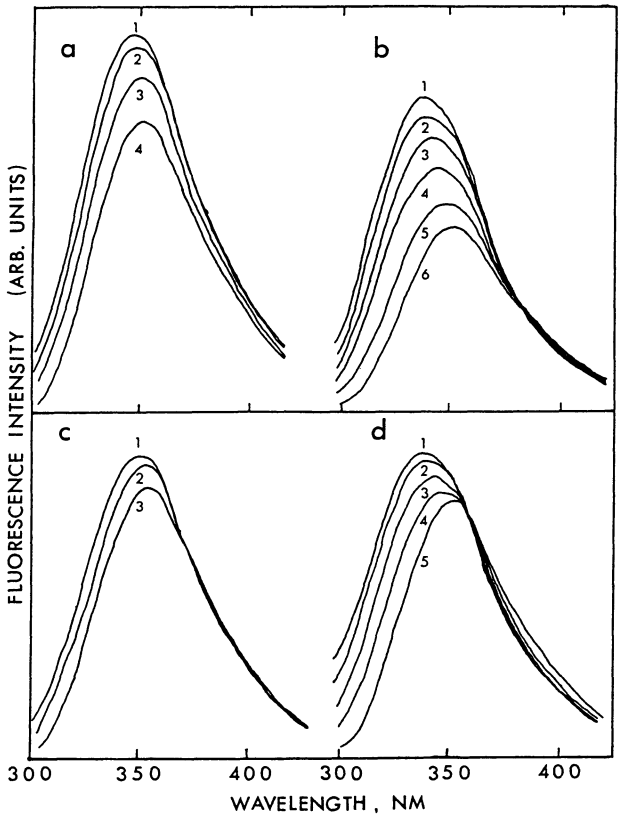

Fig. 1. Fluorescence spectra of LTL in the presence of DMPS vesicles in 20 mM Tris-acetate buffer: LTL concentration, 12.5 μM; (a) pH 5.0; 25 °C; DMPS concentration; 1, 200 μM; 2, 100 μM; 3, 50 μM; 4, 0 μM; (b) pH 5.0; 45 °C; DMPS concentration; 1, 150 μM; 2, 100 μM; 3, 75 μM; 4, 50 μM; 5, 25 μM; 6, 0 μM; (c) pH 7.0; 25 °C; DMPS concentration; 1, 150 μM; 2, 50 μM; 3, 0 μM; (d) pH 7.0; 45 °C; DMPS concentration; 1, 250 μM; 2, 200 μM; 3, 100 μM; 4, 50 μM; 5, 0 μM.

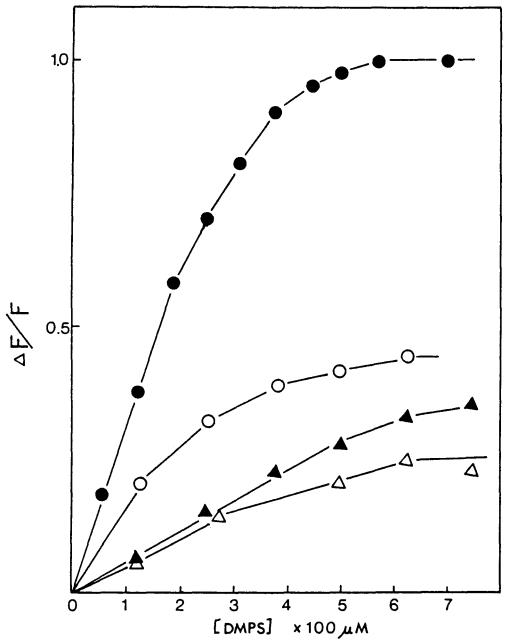

Fig. 2. Relative increase of LTL fluorescence intensity ($\Delta F/F$) on the addition of DMPS: —●—, pH 5.0 at 45 °C; —○—, pH 5.0 at 25 °C; —▲—, pH 7.0 at 45 °C; —△—, pH 7.0 at 25 °C; LTL concentration, 12.5 μM.

at different pHs and temperatures. The fluorescence spectrum of free LTL excited at 280 nm has a maximum at 352 nm. Upon addition of DMPS, the spectrum shifted to shorter wavelengths and the fluorescence intensity increased with increasing concentration of DMPS. From the variation of the fluorescence, titration curves for the binding of LTL to DMPS under various conditions were constructed by plotting $\Delta F/F$ values against the concentration of DMPS, where F is the fluorescence intensity of free LTL and ΔF is the difference in the fluorescence intensities between free and bound LTLs. As shown in Fig. 2, $\Delta F/F$ values increased with increasing concentration of DMPS and reached a plateau for all samples. It is noticeable that the titration curve was varied with pH and temperature. The $\Delta F/F$ values at pH 5.0 were larger than those at pH 7.0, and increased with increasing temperature. Ratios of the maximal fluorescence $\Delta F^{max}/F$ of the LTL-DMPS complex to that of free LTL were obtained at different pHs and temperatures. At pH 5.0 and 26 °C, the $\Delta F^{max}/F$ value was 1.45 and the emission maximum was 348 nm. The emission maximum shifted to 335 nm and the $\Delta F^{max}/F$ value increased to 2.0 with increasing temperature from 26 to 45 °C. Such shift of emission maximum and increase in $\Delta F^{max}/F$ value were also observed when measurement was done at pH 7.0. The maximum shifted from 348 to 340 nm and the $\Delta F^{max}/F$ value increased from 1.30 to 1.40. For better understanding of the nature of the binding of LTL to DMPS vesicles, experiments were done at various temperatures with an LTL-DMPS complex in which the LTL molecule was completely saturated with DMPS vesicles. As shown in Fig. 3, the fluorescence intensity of the LTL-DMPS complex decreased markedly with elevation of temperature from 25 to 36 °C, whereas the emission maximum remained unchanged. On elevation of temperature from 36 to 39 °C, the emission maximum shifted to 335 nm and the fluorescence intensity increased to the same level as at 25 °C. A further elevation of temperaure led to decrease in the fluorescence intensity without any noticeable change in the emission maximum. In the insert of Fig. 3, emission maxima of the LTL-DMPS complex at pHs 5.0 and 7.0 are plotted against temperature, in comparison with similar plots for free LTL. The emission maximum of free LTL remained unchanged when temperature was increased from 25 to 60 °C. Contrary to this, the emission maxima of the LTL-DMPS complex at pHs 5.0 and 7.0 shifted to the shorter wavelength side abruptly at 26 and 36 °C, respectively.

Figure 4 shows the temperature dependence of the

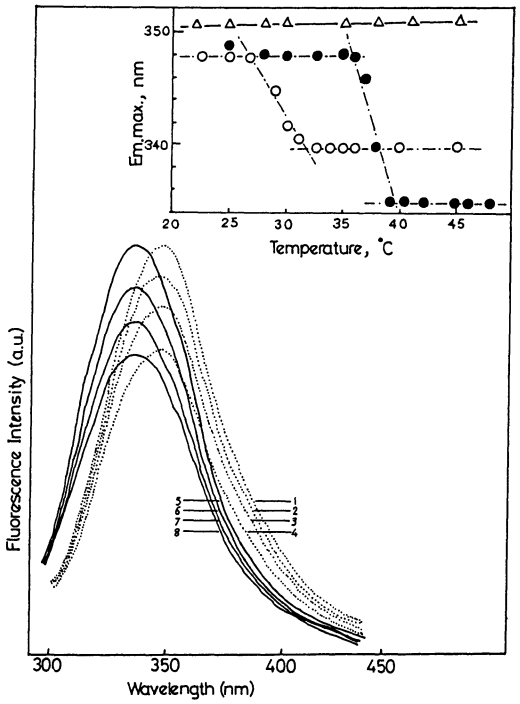

Fig. 3. Temperature dependence of fluorescence spectrum of bound LTL at pH 5.0: 1, 25; 2, 28; 3, 31; 4, 36; 5, 39; 6, 42; 7, 45; 8, 48 °C; Insert, temperature dependence of the fluorescence maximum of LTL; —△—, free LTL at pH 5.0; —○—, bound LTL at pH 7.0; —●—, bound LTL at pH 5.0.

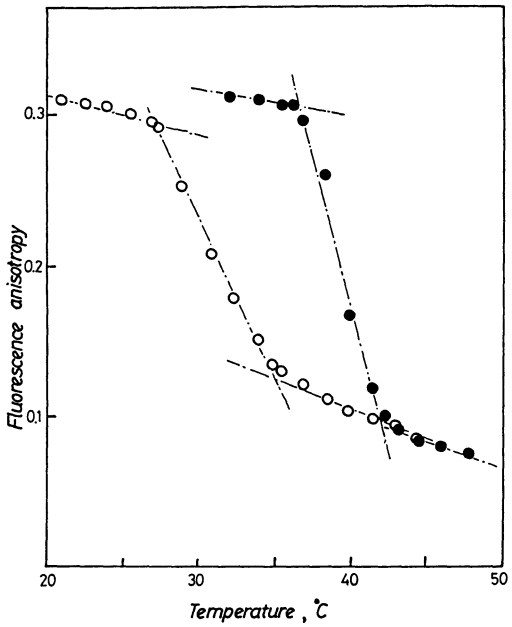

Fig. 4. Fluorescence anisotropy of DPH in DMPS vesicles: —○—, pH 7.0; —●—, pH 5.0; [DMPS]/[DPH]=1000.

fluorescence anisotropy of DPH in DMPS vesicles. When the temperature of gel-liquid crystalline phase transition (T_c) was evaluated from the midpoint of the anisotropy change as shown in Fig. 4, the T_c of DMPS was found to be 39 °C at pH 5.0 and 31 °C at pH 7.0.

Kinetics of Fluorescence Decay. The fluorescence decay kinetics of LTL was analyzed according to the equation

$$F(t)=\sum_j A_j \exp(-t/T_j), \quad (1)$$

where $F(t)$, A_j, and T_j are the fluorescence intensity at t, normalized preexponential factor, and lifetime of the jth component, respectively. The fluorescence decay of LTL in the presence of DMPS vesicles obeys the double exponential kinetics. As shown in Fig. 5, an analysis using the double exponential kinetics has

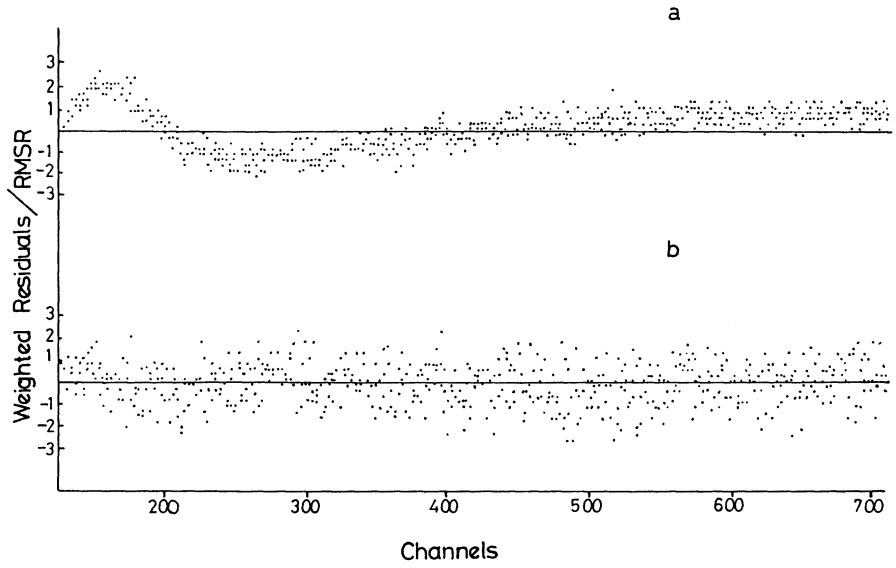

Fig. 5. Plots of weighted residuals after iterative convolution of the fluorescence decay of the bound LTL ([DMPS]/[LTL]=50, 30 °C, pH=5.0) for the best fits: (a) single exponential decay function; (b) double exponential decay function. E_m=335 nm, 22 ps/channel.

Table 1. Kinetic Parameters of the Fluorescence Decay of LTL

	pH	Temp/°C	T_1/ns[a)]	T_2/ns[a)]	A_1	A_2
Buffer	5.0	26	2.24	0.90	0.51	0.51
		40	1.63	0.67	0.47	0.53
DMPS	5.0	26	2.69	0.75	0.69	0.31
		40	2.06	0.60	0.60	0.40
Buffer	7.0	26	2.50	1.20	0.72	0.28
		40	1.90	1.16	0.57	0.43
DMPS	7.0	26	0.56	0.82	0.51	0.49
		40	1.92	0.64	0.45	0.55

a) Standard error=0.02 (for T_1), and 0.03 (for T_2), the weighted root mean square residuals (RMSR) of all measurements are within 1.0±0.1. RMSR=$[1/N\sum_i^C 1/R_{ie}\ (R_{ie}-R_{ic})^2/R_{ie}]^{1/2}$, where N is the number of degrees of freedom, C is the number of channels, and R_{ie} and R_{ic} are, respectively, the experimental and calculated numbers of counts in the ith channel.

revealed that the weighted residuals (RMSR) distribute homogeneously and that the root mean square residuals are nearly 1.0 (panel b), whereas the single exponential analysis gives an extremely heterogeneous distribution of the residuals (panel a). The decay of free LTL also perfectly fits to the double exponential kinetics. The resulting decay parameters are summarized in Table 1. The two preexponential factors (A_1 and A_2) of LTL, whether free or bound, are positive and the emission wavelength dependence of the decay parameters could not be observed over the entire fluorescence spectrum region (310—450 nm). The temperature dependence of the lifetime of free

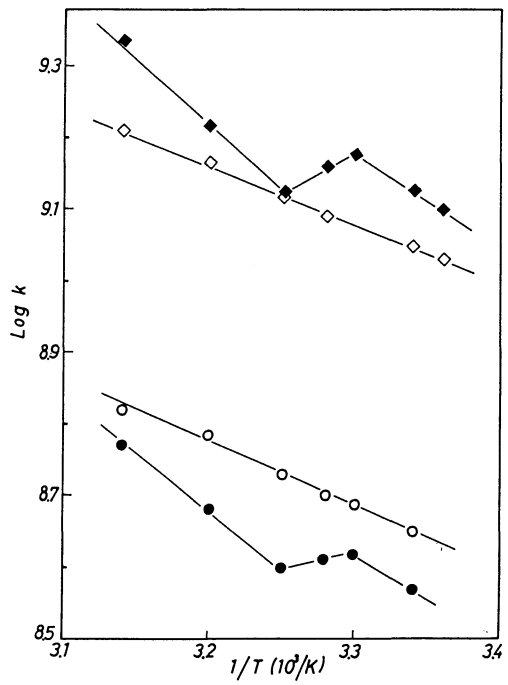

Fig. 6. Temperature dependence of the fluorescence decay constant of LTL at pH 5.0: —◆—, short lived component of bound LTL; —◇—, short lived component of free LTL; —●—, long lived component of bound LTL; —○—, long lived component of free LTL; [DMPS]/[LTL]=50; $k=1/T_1$, $1/T_2$.

LTL is different from that of the LTL-DMPS complex. When $1/T_1$ and $1/T_2$ of free LTL are plotted against $1/T$, straight lines are obtained as shown in Fig. 6. On the other hand, the lifetime of bound LTL was prolonged temporally at the phase-transition temperature of DMPS vesicles. Although the prolongation of the two lifetimes of bound LTL at pH 7.0 at the phase transition temperature was not so large as at pH 5.0, clear deviation from a linear line is observed with the plots of $\ln[1/T_1, 1/T_2]$ against $1/T$. The fluorescence decays of free and bound LTL can be described in terms of the same type of kinetics (double exponential kinetics). But the decay parameters of free LTL are modified by the binding with DMPS vesicles. A comparison of the lifetime of free LTL with that of bound LTL has revealed that at each temperature in the presence of vesicles the longer lifetime is prolonged, the shorter becoming still shorter. This suggests a peculiar interaction of LTL with DMPS vesicles.

Solute Quenching of Tryptophan Fluorescence. The fluorescence intensity of bound LTL was measured in the presence of acrylamide, and quenching data were analyzed in accordance with the Stern-Volmer plot based on the equation

$$F_0/F=1+k_qT_{av}[Q] \quad (2)$$

where F_0 and F are the fluorescence intensities in the absence of acrylamide and in its presence at concentration [Q], respectively, k_q is the quenching constant, and T_{av} is the weighted mean lifetime of bound LTL which is defined as $T_{av}=(A_1T_1+A_2T_2)/(A_1+A_2)$. In the plots of F_0/F versus the concentration of acrylamide, straight lines were obtained for all the samples as shown in Fig. 7. In Fig. 8, quenching constants (k_q) are plotted against temperature. Quenching constants of free LTL increase linearly with elevation of temperature. However, reflection points are found at the T_c of DMPS (29 °C at pH 7.0 and 38 °C at pH 5.0) in the plots given for the LTL-DMPS complex, and k_q values increase above the phase transition

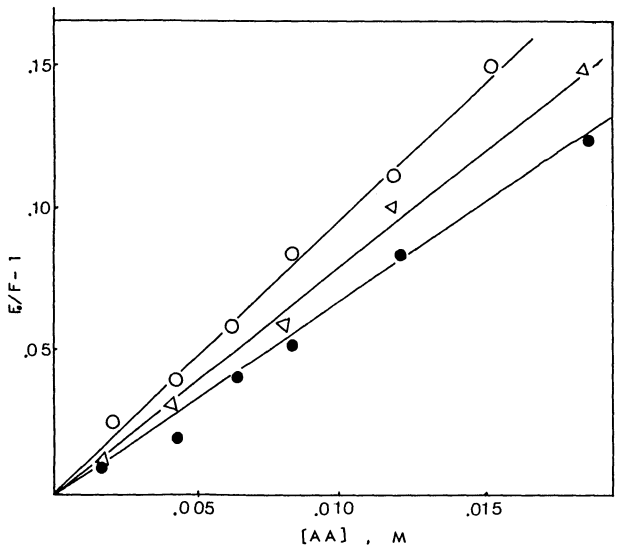

Fig. 7. Stern-Volmer plots for the quenching of the fluorescence of the bound LTL by acrylamide at pH 5.0: —○—, 30 °C; —●—, 40 °C; —△—, 45 °C.

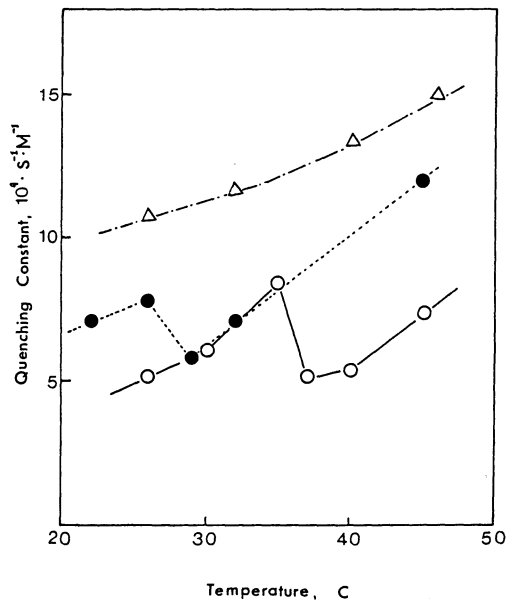

Fig. 8. Temperature dependence of quenching constant for the fluorescence of bound and free LTL: ---△---, free LTL at pH 5.0; —○—, bound LTL at pH 5.0; ---●---, bound LTL at 7.0; [DMPS]/[LTL]=50; Weighted mean lifetimes were used for evaluating the quenching constant (see text).

temperatures.

Discussion

The results presented here provide some insight into the property of the binding of LTL, a tripeptide, to DMPS vesicles. In this work, the intrinsic fluorescence of LTL due to the tryptophan residue was advantageously utilized to analyze the peptide-lipid interaction. As demonstrated here, the binding of LTL to DMPS vesicles induces a shift of the fluorescence emission maximum and change in the fluorescence intensity of LTL (Fig. 1). Such a change in the fluorescence spectrum of LTL can be interpreted as being due to a change in the environment of the tryptophan residue caused by the binding with DMPS vesicles. It is noteworthy that the interaction of LTL with DMPS vesicles, resulting in change in fluorescence spectrum, is greatly influenced by temperature (Fig. 2). Below the phase-transition temperature of DMPS vesicles, the binding of LTL to DMPS induced only a slight shift of emission maximum, whereas large shift of emission maximum was observed above the phase-transition temperature (Fig. 3). Such a difference in the fluorescence spectra of the LTL-DMPS complex observed below and above T_c is attributable to the difference in the behavior of the tryptophan residue in the phospholipid between the gel and liquid crystalline phases. It is postulated that in the temperature range below the T_c of DMPS vesicles, the indole side chain of the tryptophan residue of the peptide would slip into the environment of the acyl chain moiety of DMPS vesicles which is less polar than that in the aqueous medium, but that it would still remain near the head group, i.e., the phosphatidylserine moiety of the DMPS molecule. It is plausible that in the liquid crystalline phase of DMPS vesicles, the packing of the phospholipid is less dense than in the gel phase, which facilitates the penetration of the indole side chain of tryptophan into DMPS vesicles. Such a view is consistent with our previously postulated model in which the gel phase of phospholipid is so tightly packed as to prevent the entry of the indole ring into the acyl moiety of the phospholipid bilayer. NMR studies on lipid-water systems[8,9] have indicated that phosphatidylserine has a rigid head group as compared to phosphatidylcholine and phosphatidylethanolamine, and that the distance of head groups between DMPS molecules in the vesicles is made longer when the phase transition of vesicles occurs. In the solute quenching experiment, the quenching constant for tryptophan fluorescence in the LTL-DMPS complex was reduced at the phase-transition temperature of DMPS vesicles both at pHs 5.0 and 7.0 (Fig. 4). Such a change in the quenching constant as observed at T_c might be explained by change in the distance between head groups in DMPS molecules in vesicles as suggested by NMR measurements.

The phase-transition temperature of DMPS evaluated from the fluorescence anisotropy of DPH is 39 °C at pH 5.0 and 31 °C at pH 7.0 (Fig. 4). These values are in good agreement with those obtained from phase-transition titration curves using spin probe.[10] However, the apparent transition temperatures estimated from the shift of the emission maximum of the LTL-DMPS complex are lower than the T_c values obtained by the fluorescence anisotropy of DPH (the

insert in Fig. 3). The discrepancy of the transition temperatures of DMPS vesicles observed between the two methods may be due to perturbation to the order of the acyl chains in DMPS vesicles caused by the binding with LTL. It has been reported that an electrostatic binding of positively charged molecules to DMPS led to a marked increase in the T_c of phospholipid.[11] In the present case, however, the T_c of DMPS was reduced after DMPS's binding with LTL. There may be differences in modes of binding to phospholipid between LTL and other cationic molecules.

The time resolved fluorescence study also supports the penetration of the tryptophan residue into deeper sites of the membrane on phase transition. The prolongation of the fluorescence lifetime on phase transition is coincident with the solvent polarity dependence of the fluorescence lifetime of indole which is the chromophore of tryptophan residue. The mean lifetime of indole in nonpolar solvent is longer than in aqueous solution, although the radiative lifetime is almost the same.[12]

The double exponential decay kinetics from a single fluorophore arises from the existence of a ground state equilibrium, since the preexponential terms are both positive and their ratio is nearly constant over the entire fluorescence spectrum of LTL. It is confirmed that three rotamers around C_α-C_β of tryptophan residue are responsible for the multiexponential fluorescence decay since they give heterogeneous microenvironments to LTL in association with the decay of the excited singlet state. The indole rings of two rotamers (rotamers I and III) are adjacent to the C_α-carbonyl group and -H or -NH. The other rotamer (rotamer II) has a structure such that the indole is not close to the carbonyl group. C_α-Carbonyl group is an efficient intramolecular quencher in indole derivatives (A. G. Szabo and D. T. Krajcarski, unpublished observation). According Szabo et al.,[12,13] therefore, the longer lived component can be assigned to rotamer II, and rotamers I and III account for the shorter lifetime. The dual fluorescence emission can be rationalized if the quenching efficiences of rotamers I and III are similar to each other or if their interconversion is very rapid. On the basis of the above assignment, the biphasic effect (prolongation of T_1 and shortening of T_2) of the binding on the fluorescence lifetimes of LTL suggests a peculiar interaction between LTL and DMPS. It is clear that the prolongation of T_1 of free LTL by the binding with DMPS is caused by the decrease in polarity of the environment around the indole side chain. The indole side chain of T_2 component also must be under the same conditions. However, clear shortening of T_2 was observed to be caused by the binding with DMPS. This suggests that some quenching interaction which works effectively for rotamer I and III is created by the binding with DMPS. For such specific interaction, we postulate a structural distortion of LTL resulting in the enhancement of the quenching efficiency of C_α carbonyl group. The indole ring could be more accessible to the carbonyl group by the distortion induced by the binding. Since the quenching by the carbonyl group is not involved in the decay channel of rotamer II, the fluorescence lifetime of rotamer II is free from such an effect. Some contribution due to the intermolecular quenching by DMPS should be examined closely. But it seems unreasonable, because the quenching by any groups of DMPS such as carboxyl group is not so efficient as carbonyl group. If DMPS acted as an efficient quencher, we could not observe the increase in the fluorescence quantum yield of LTL with the binding with DMPS as shown in Figs. 1 and 2.

The steady state and time resolved fluorescence studies have consistently described the phase dependence of the interactions between LTL and DMPS vesicles. Further, it has been suggested that structural change of LTL is induced by the binding with the membrane. The present results can give no fine detailed structure of LTL in the membrane. But increase of our knowledge on the photophysics of tryptophan by means of improved instrumentation could contribute to solving the problems we have proposed here.

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