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Kinetics near the pretransition of a multilamellar phospholipid studied by ESR

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The kinetics of the pretransition were studied by electron spin resonance (ESR) of the spin probe, TEMPO, in multilamellar dipalmitoylphosphatidylcholine liposomes. The temperature was jumped from the $P_{\beta'}$ phase to the $L_{\beta'}$ phase and vice versa. The relaxation process to the equilibrium state at fixed temperature was obtained by measuring the change of the peak height of the ESR line with time. It was clearly established that both the relaxation processes, $P_{\beta'} \rightarrow L_{\beta'}$ and $L_{\beta'} \rightarrow P_{\beta'}$, were well described by the sum of the fast and the slow relaxation times, t_1 and t_2, in each relaxation process were determined by a least-squares fitting analysis at various fixed temperatures near the pretransition temperature T_p. The slow relaxation time, t_2, is more than one order longer than the fast relaxation time, t_1. Both the relaxation times, t_1 and t_2, in the cooling experiment are larger than those in the heating experiment. The logarithm of t_1 and t_2 increases towards T_p almost linearly against temperature.

Introduction

The thermal hysteresis of the pretransition in multilamellar phospholipid liposome has been studied by various methods [1–3]. Lentz et al. [4] concluded from fluorescence depolarization studies that hysteresis resulted from the kinetic nature of the pretransition. The kinetic behavior of the pretransition was intensively studied in detail by Cho et al. [5] for two phospholipids, dipalmitoylphosphatidylcholine (DPPC) and dimyristoylphosphatidylcholine (DMPC), by using differential scanning calorimetry (DSC). They measured the transition half-time, which is the characteristic time taken to reach the equilibrium state, by the following methods. Samples in the $P_{\beta'}$ phase were rapidly cooled to a preset temperature in the $L_{\beta'}$ phase and kept at that temperature for a certain number of minutes. Then, the DSC measurements were carried out in the heating scan and the area under the pretransition curve was assumed to be proportional to the part transformed to the $L_{\beta'}$ phase. The dependence of the area under the pretransition curve on the annealing time was estimated. The opposite transformations from the $L_{\beta'}$ phase to the $P_{\beta'}$ phase were also studied by using more complicated temperature scan procedures. They indicated that the transition half-time became longer near the pretransition temperature, T_p. They concluded that the transition from the $P_{\beta'}$ phase to the $L_{\beta'}$ phase was not given by a simple relaxation process but a relaxation process via metastable states. Contrary to the DSC experiment, the peak which corresponds to the pretransition disappears in the a.c. calorimetry experiment at about 1 Hz [6]. This fact also suggests the presence of the long relaxation time at the pretransition. Recently, the relaxation behavior observed

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by small-angle X-ray diffraction was reported by Akiyama et al. [7] for DMPC. they showed that the transition from the $P_{\beta'}$ phase to the $L_{\beta'}$ phase is complete in 7 min after the temperature jump, but that some imperfection of the multilamellar structure remains for a long time. However, the characteristic times obtained from the various methods do not coincide with each other and the kinetics of the pretransition has not yet been explained so clearly.

In the present experiment, we determined the relaxation time accurately. The temperature was rapidly changed and fixed at each preset temperature. Then, the peak height of the ESR line was measured as a function of time. The relaxation curve to the equilibrium state was observed directly, because the present experiment did not need the scanning time or the indispensable time for carrying out the measurement, in comparison with the other experiments.

Methods

The lipid DPPC which is more than 99% pure was purchased from Sigma. Its purity was established by thin-layer chromatography and was used without further purification. The lipid was dissolved in chloroform and the solution was dried under a stream of nitrogen gas. The remaining lipid was kept in an evacuated desiccator for at least 1 h to remove residual solvent. Distilled water was added to the lipid. The concentration of the lipid was 20 wt%. This sample was kept at about 55°C for 10 h to obtain a homogeneous suspension. Then, the spin label, TEMPO, was added at a concentration of about $5 \cdot 10^{-4}$ M. The suspension was vortexed for 5 min and put into a sample tube for ESR experiments. The size of the sample cell was 1.2 mm in diameter and 2.0 mm in length. The calibrated copper-constantan thermocouple was set at the center of the sample in the cell to measure the temperature. The ESR spectra were observed at the frequency of an X-band with a JEOL FE-1X type ESR spectrometer. The temperature was controlled within an accuracy of about 0.1°C.

Fig. 1 shows an example of the first derivative of the ESR spectra for TEMPO with respect to te. magnetic field in the $P_{\beta'}$ phase. The lines designated A and B correspond to TEMPO in the lipid and in water, respectively. The linewidth of these lines was confirmed to be constant during the course of the experiment. Therefore, h relates to the amount of TEMPO in lipid and x in water. The TEMPO partition function f is usually defined by $f = h/(h+x)$ [8]. Since the total amount of TEMPO is constant over the present measuring period of about 2 h, the denominator $h+x$ is regarded to be constant. Then, the peak height h is proportional to the TEMPO partition function in the course of the experiment. The magnetic field of the ESR spectrometer was fixed to the peak of the line A. If the position of the peak shifted upon measuring the value of h, the magnetic field was corrected so as to point the peak. The temperature was rapidly varied beyond the pretransition temperature, fixed to preset temperatures and the time dependences of h were measured.

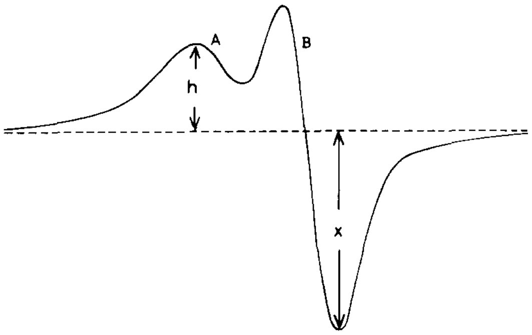

Fig. 1. An example of the first derivative of the high-field portion of the ESR spectrum for the TEMPO signal in the $P_{\beta'}$ phase of DPPC.

The time in which TEMPO diffuses into or out the lipid can be estimated from the response of h after temperature jump beyond the main transition and this time was measured to be within a few seconds. This time is also confirmed from the fact that the temperature hysteresis of the main transition between the rapid heating and cooling (0.4 deg. C/s) is about 1°C as shown later in Fig. 2.

Results

First, the temperature dependence of the peak height, h, measured under various temperature-scanning rates is shown in Fig. 2. The peak height

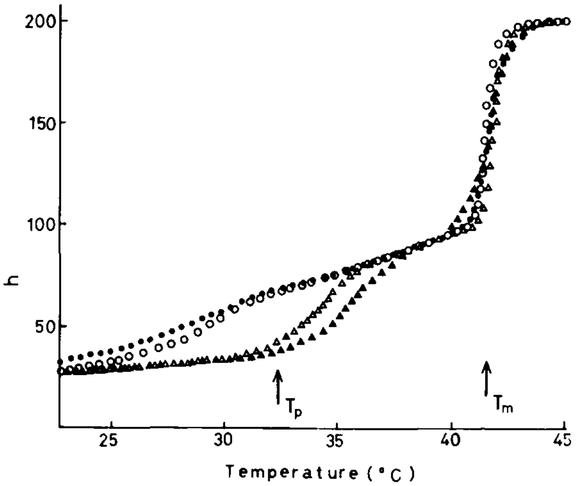

Fig. 2. The temperature dependence of the peak height, *h*. Scanning rate is: (Δ) 0.01 deg. C/s (heating); (▲) 0.4 deg. C/s (heating); (○) 0.01 deg. C/s (cooling); (●) 0.4 deg. C/s (cooling).

was normalized to 200 in arbitrary units at 43°C, because the peak height is almost independent of the scanning rate at 43°C. The temperature dependence of *h* agrees with that reported by other authors [8]. The abrupt increase of *h* near 41°C is due to the main transition, and the gradual increase between 25 and 35°C is due to the pretransition. The remarkable hysteresis observed at the pretransition depends on the temperature-scanning rate, as in the other experiments [1–4]. The hysteresis of the pretransition is schematically shown in Fig. 3 in an expanded scale. The middle thin line corresponds to the equilibrium value of *h*, which was determined in the following way in the heating process. The temperature was fixed at a preset temperature after rapid heating along the thick line and the limiting value of *h* after 2 h was defined to be the equilibrium value at the fixed temperature. It will be confirmed from the analysis of the relaxation time that this interval is long enough to reach the equilibrium value. The pretransition temperature, T_p, determined from the point with the steepest slope of the middle thin line is 32.5 ± 0.2°C. Δh_0 indicates the difference between the initial value and the equilibrium value and Δh indicates the difference between the transient value and the equilibrium value. The temperature was changed as denoted by arrows in Fig. 3.

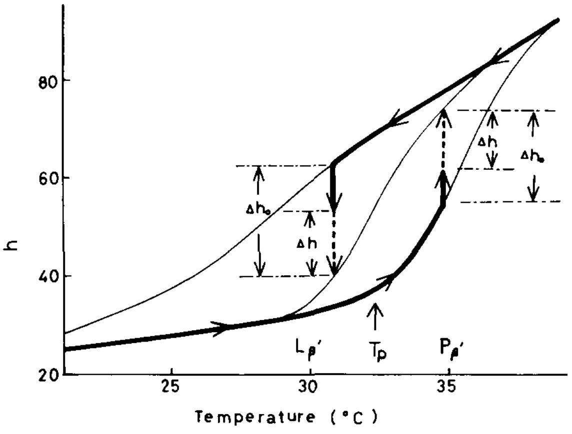

Fig. 3. Schematical view of the hysteresis at the pretransition. The upper line corresponds to the rapid-cooling scan at the rate of about 0.4 deg. C/s and the lower line corresponds to the rapid-heating scan at the rate of about 0.4 deg. C/s. The middle line corresponds to the value in the equilibrium state. Temperature was changed as denoted by arrows. In the process $P_{\beta'} \rightarrow L_{\beta'}$, The temperature was quickly decreased along the upper thick curve down to the temperature in the $L_{\beta'}$ phase and the relaxation along the vertical thick-and-dotted line was the process for measuring $\Delta h(t)$. In the process, $L_{\beta'} \rightarrow P_{\beta'}$, temperature was quickly increased along the lower thick curve up to the temperature in the $P_{\beta'}$ phase and the relaxation along the vertical line was observed as well. The transition temperature was defined to be the point where the slope of the equilibrium line was the steepest as 32.5 ± 0.2°C.

In the experiment on the heating process, each sample was initially kept at 10°C and rapidly heated at a rate of about 0.4°C/s and then fixed at a temperature above T_p. Fig. 4 shows the time dependences of $\Delta h/\Delta h_0$ at the various temperatures. As seen in Fig. 4, *h* decays more gradually as the temperature approaches T_p. Near T_p, it takes about 2 h to reach the equilibrium value. On the other hand, *h* reaches the equilibrium value within 4 min at 35.7°C. Fig. 5 shows the time dependences of $\Delta h/\Delta h_0$ in the cooling process. In this case, each sample was initially set for several minutes above the main transition temperature and then kept at 38°C for about 10 min. After this procedure, it was rapidly cooled at the rate of about 0.4 deg. C/s and fixed at the temperature below T_p. A fairly long time is in principle required to reach the equilibrium value of *h* in the cooling process. Then, the equilibrium value was determined in this case by the following method.

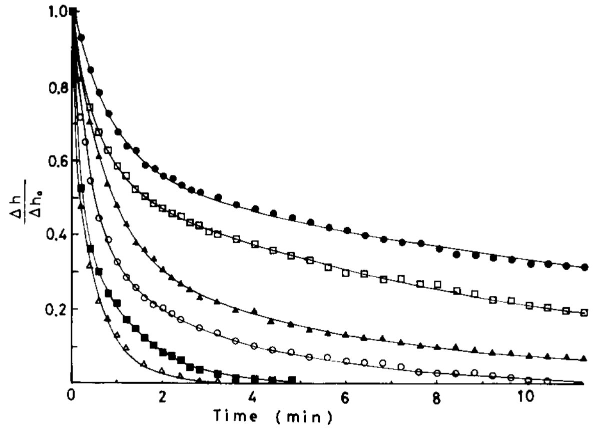

Fig. 4. time depencence of $\Delta h/\Delta h_0$ in the heating process $L_{\beta'} \rightarrow P_{\beta'}$. The fixed temperatures (°C) are (●) 32.6; (□) 33.0; (▲) 33.5; (○) 34.4; (■) 35.0; (△) 35.7. The curves fitted to the data points indicate the results of the least-squares fitting analysis.

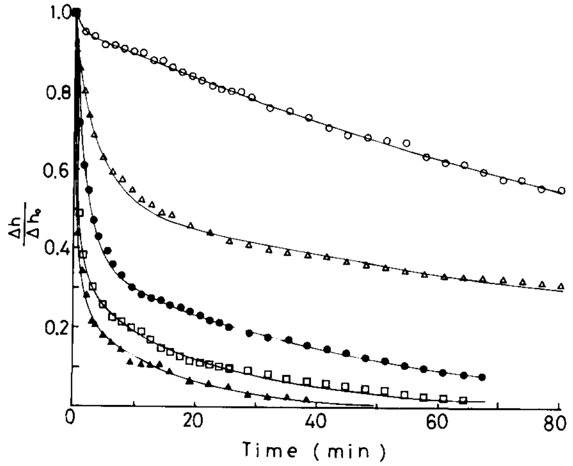

Fig. 5. Time dependence of $\Delta h/\Delta h_0$ in the cooling process $P_{\beta'} \rightarrow L_{\beta'}$. The fixed temperatures (°C) are: (○) 31.7; (△) 30.8; (●) 30.0; (□) 28.6; (▲) 27.9. The curves fitted to the data points indicate the results of the least-squares fitting analysis.

The sample was cooled further down to 10°C, after measuring h, for about 2 h and then heated up to the measuring temperature again. Then, the limiting value, after this procedure, was assumed to be the equilibrium value at this fixed temperature.

The relaxation process of $\Delta h/\Delta h_0$ shown in Figs. 4 and 5 cannot be described by a single relaxation process. So, two processes, fast and slow relaxation, were introduced as follows:

$$\Delta h/\Delta h_0 = a \exp(-t/t_1) + b \exp(-t/t_2). \qquad (1)$$

A least-squares fitting analysis was performed by using the SALS program of the Nagoya University Computation Center, treating a, b, t_1 and t_2 as adjustable parameters. The best fitted values are listed in Table I. Thus, calculated values of $\Delta h/\Delta h_0$ from these parameters are drawn by solid lines in Figs. 4 and 5. The temperature dependences of the relaxation times, t_1 and t_2, are plotted in Fig. 6.

From the above table and figures, we can conclude as follows. From Figs. 4 and 5, the relaxation feature is well described by the two relaxation processes. Both $\log t_1$ and $\log t_2$ exhibit almost

TABLE I

PARAMETERS IN EQN. 1 DETERMINED BY THE LEAST-SQUARES FITTING ANALYSIS IN THE HEATING PROCESS $L_{\beta'} \rightarrow P_{\beta'}$ (A) AND THE COOLING PROCESS $P_{\beta'} \rightarrow L_{\beta'}$ (B)

	Temperature (°C)	a	b	t_1 (s)	t_2 (s)
A	35.7	0.34 ± 0.17	0.66 ± 0.17	3 ± 3	36 ± 8
	35.0	0.52 ± 0.12	0.48 ± 0.12	8 ± 7	75 ± 20
	34.4	0.60 ± 0.13	0.40 ± 0.13	23 ± 7	170 ± 60
	33.5	0.62 ± 0.06	0.38 ± 0.06	43 ± 10	340 ± 50
	33.0	0.42 ± 0.04	0.55 ± 0.04	39 ± 25	620 ± 30
	32.6	0.45 ± 0.04	0.55 ± 0.03	58 ± 12	1190 ± 120
B	31.7	0.04 ± 0.05	0.96 ± 0.03	–	8700 ± 1000
	30.8	0.45 ± 0.04	0.52 ± 0.03	230 ± 50	8500 ± 1300
	30.0	0.62 ± 0.05	0.38 ± 0.03	120 ± 20	2600 ± 300
	28.6	0.68 ± 0.05	0.32 ± 0.03	38 ± 7	1300 ± 200
	27.9	0.73 ± 0.07	0.28 ± 0.07	39 ± 8	800 ± 300

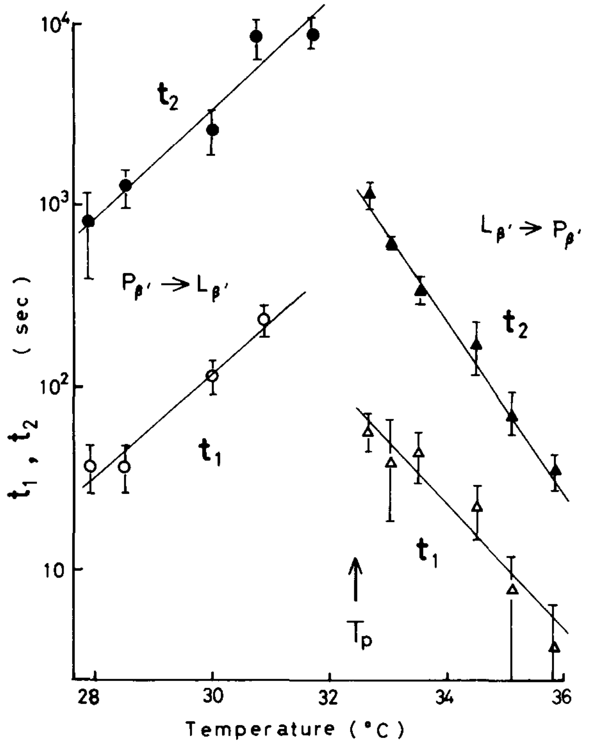

Fig. 6. Temperature dependences of the relaxation times, t_1 and t_2: (●) t_2 in the cooling process; (○) t_1 in the cooling process; (▲) t_2 in the heating process; (△) t_1 in the heating process. The solid lines were not calculated and are included only to guide the eye.

linear increase near T_p as shown in Fig. 6. Table I shows that both t_1 and t_2 in the cooling process are much longer than those in the heating process, respectively, and that the slow relaxation time t_2 is more than one order longer than the fast relaxation time t_1 in each process. The partitions of both fast and slow relaxations, *a* and *b*, have a comparable order. However, the partition of the slow relaxation, *b*, increases greatly near T_p in the cooling process.

Discussion

The kinetic behavior of the pretransition of DPPC and DMPC has been studied by Cho et al. [5] by using DSC methods. The relaxation process appearing after a temperature jump from high temperature, $P_{\beta'}$ phase to low temperature, $L_{\beta'}$ phase ($P_{\beta'} \rightarrow L_{\beta'}$) and the opposite relaxation process ($L_{\beta'} \rightarrow P_{\beta'}$) were observed. The transition half-times were measured at various temperatures. They showed that the transition half-time increases logarithmically near T_p. They suggested that the relaxation process, $P_{\beta'} \rightarrow L_{\beta'}$ took place via a metastable state M, and the fast, $P_{\beta'} \rightarrow M$, and the slow, $M \rightarrow L_{\beta'}$ processes appear. Although the DSC data indicate that the process $L_{\beta'} \rightarrow P_{\beta'}$ was a simple relaxation process, it was revealed in the present experiment that both the processes $P_{\beta'} \rightarrow L_{\beta'}$ and $L_{\beta'} \rightarrow P_{\beta'}$ were well described by the two relaxation processes. Moreover, there exist quantitative differences in the characteristic time between these experiments. Especially, the characteristic time of the process $L_{\beta'} \rightarrow P_{\beta'}$ in the present experiment is much shorter than that of the DSC measurement. The reason for these facts is explained as follows. The DSC experiment on the relaxation process $L_{\beta'} \rightarrow P_{\beta'}$ needs a very complicated temperature-scanning process, and the fast relaxation process which takes place within 1 min might then be missed and the transition half-time about only the slow relaxation process might be observed in the DSC experiment. Lentz et al. [4] for DPPC and Akiyama et al. [7] for DMPC reported longer relaxation times in the $P_{\beta'} \rightarrow L_{\beta'}$ process than in the $L_{\beta'} \rightarrow P_{\beta'}$ process. Akiyama et al. suggest that the above result is due to the difference in fluidity between the two phases.

The kinetics of the pretransition in multilamellar DMPC was intensively studied by Akiyama et al. [7] by using the small-angle X-ray diffraction method. They reported that the transformation from the $P_{\beta'}$ phase to the $L_{\beta'}$ phase was 90% completed at 7 min after a temperature jump, but the width of the reflection decayed slowly with a longer relaxation time. By our estimation from Figs. 2 and 3 of Ref. 7, it can be found that the integrated intensity of the $L_{\beta'}$ component is recovered with the relaxation time of about 1 min and the width decays with the relaxation time of about 25 min. Although the sample and the transition temperature are different from those of the present experiment, the magnitude of the two relaxation times in this X-ray experiment qualitatively coincides with the present experiment: $t_1 = 0.6$ min, $t_2 = 22.0$ min at 28.6°C. Very recently, Akiyama and Terayama [9] carried out a similar X-ray experiment for DPPC. They found that the ripple structure in the $P_{\beta'}$ phase appears immediately after the heating procedure of $L_{\beta'} \rightarrow P_{\beta'}$ and on the other hand, disappears immediately after the cooling procedure of $P_{\beta'} \rightarrow L_{\beta'}$ within the time resolution of their experiment. Furthermore, the width of reflection associated with the fluctuation of the multilamellar layer distance decays with a long relaxation time. In connection with these facts, the relaxation time of the peak intensity was found to be about 130 min at 30°C in the process $P_{\beta'} \rightarrow L_{\beta'}$. This coincides with the present slow relaxation time at 30.8°C. Therefore, it is very likely that the fast relaxation mechanism is due to the creation or the extinction of the ripple structure and the slow relaxation mechanism is due to the fluctuation of the layer spacing.

The peak height h of the line A should be revised by the change of the linewidth in order to correspond to the real intensity of the line A, although the width was assumed to be constant in the present experiment. The width of the line A, which could be estimated from the halfwidth of the first derivative line, decreases slightly about 10% upon increasing the temperature from 27 to 36°C. The revision of the peak height h due to this change of the width is small and almost linear in temperature within the range of the present experiment and, moreover, the normalized value $\Delta h/\Delta h_0$ was used in the analysis of the relaxation processes. These facts suggest that the change of the width has no considerable influence on the present calculation of the relaxation times t_1 and t_2.

Finally, it is worthwhile pointing out that the possibility for a freeze-fracture experiment depends largely on the long relaxation time at the pretransition. Therefore, the condition of the freezing for the $P_{\beta'}$ phase is not simply applicable to that of the L_α phase, becasue the characteristic relaxation time is completely different between the main transition and the pretransition. This means that it is comparatively difficult to freeze the structure of the L_α phase.

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