Biochimica et Biophysica Acta 898 (1987) 53–58
Elsevier

BBA 73413

Dynamics of appearance and disappearance of the ripple structure in multilamellar liposomes of dipalmitoylphosphatidylcholine

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(Received 27 November 1986)

Key words: Phase transition; Freeze-fracture electron microscopy; Dipalmitoylphosphatidylcholine; Relaxation; Multilamellar liposome

The physical properties of the pretransition ($P_{\beta'} \leftrightarrow L_{\beta'}$) of dipalmitoylphosphatidylcholine liposomes were investigated using freeze-fracture electron microscopy. The kinetics of pretransition examined in the previous paper using TEMPO spin probe (Tsuchida, K., et al. (1985) Biochim. Biophys. Acta 812, 249–254) was extensively studied by observing the ripple structures in the freeze-fractured surfaces at different time intervals. When the temperature is decreased from 38°C to 30°C, the ripple structure disappears in the following steps. The intervals between ripples begin to expand with the decrease of ripple density upon the temperature shift, and this process continues for several tens minutes. Then, each ripple disappears gradually and changes into a completely smooth surface at 3 h after the temperature shift. The comparison of relaxation times between the previous ESR measurement and the present experiment suggests that the fast relaxation observed in the previous study corresponds to the expansion of the intervals between ripples. On the other hand, the ripple structure of regular intervals appears rapidly in some places and then spreads over the whole area of fractured surface when the temperature is increased from 23°C to 35°C. The results obtained in this work and the previous ESR work strongly suggest that the formation and disappearance of ripple structure is closely related to the relaxation processes near the pretransition temperature.

Introduction

Phosphatidylcholine is one of the phospholipids studied most intensively. The reason for this is that phosphatidylcholine is a major constituent of membranes of most living organisms. Much of the interest in phospholipid research has focused on the physical properties of membranes.

It is known that there are several types of lipid phase transitions; main transition, pretransition, subtransition and bilayer-nonbilayer transition. Some of these transitions seem to be closely related to the functions of biological membranes. Phase transition between solid and fluid phases is a major physical property of lipid bilayer membranes. In addition to the main transition, the kinetics of the pretransition has been studied in multilamellar phospholipid liposomes by various methods [1–4]. Using differential scanning calorimetry (DSC), Cho et al. [2] intensively studied the relaxation behavior of the pretransition in dipalmitoylphosphatidylcholine (DPPC)

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liposomes and dimyristoylphosphatidylcholine (DMPC) liposomes. They estimated the transition half-times around the pretransition temperature, T_p. It was shown that the transition half-time becomes longer near the pretransition temperature, and that the transition from the $P_{\beta'}$ phase to the $L_{\beta'}$ phase cannot be analyzed with a single relaxation process. Akiyama and Terayama [3] examined the process with DMPC using X-ray diffraction. They have found that the transition from the $P_{\beta'}$ phase to the $L_{\beta'}$ phase is 90% complete within 7 min after a temperature shift; however, the reflection becomes sharp with a longer relaxation time. They have concluded that some imperfection in the multilamellar structure causes a long relaxation time. Recently, the defects in the ripple structure were studied by Rüppel and Sackmann [5] in DMPC bilayers using freeze-fracture electron microscopy. They classified defects in the $P_{\beta'}$ phase topologically and analyzed the defects in terms of the homotopy theory. They have also reported that the defect of structure in the $L_{\beta'}$ phase depends on the time interval between the temperature shift and the observation, and assumed that the ripples of the $P_{\beta'}$ phase are transformed into a step-like profile after a healing time of some 10 min. Furthermore, after many hours of healing time, the surface of the bilayer becomes essentially smooth.

In our previous paper [4], the kinetics of the pretransition was studied by electron spin resonance (ESR) of the spin probe, TEMPO, in multilamellar DPPC liposomes. The liposomes were brought from the $P_{\beta'}$ phase to the $L_{\beta'}$ phase and vice versa by temperature jumps. The relaxation process to the equilibrium state at a fixed temperature after the temperature shift was monitored by measuring the change in the partition of TEMPO between the aqueous and lipid phases. The two relaxation times were determined by least-squares fitting analysis at various temperatures near the pretransition, T_p. Both times were found to become longer as the temperature approaches T_p.

In the present study, freeze-fracture electron microscopy was used to investigate multilamellar DPPC liposomes under conditions similar to that used in the ESR measurement. The time-course of formation or disappearance of the ripple structure was observed in order to study the relaxation process near T_p more extensively. The temperature of DPPC dispersions was lowered rapidly from 38°C to 30°C, and the structures of freeze-fractured surfaces were observed after various incubation times. Observations were also carried out in DPPC dispersions, the temperature of which was raised from 23°C to 35°C. The process of formation or disappearance of the ripple structure was analyzed in detail. The relation between the present freeze-fracture electron microscopic observations and the previous ESR measurement will be discussed with respect to the relaxation process. From the data of the present experiments we have concluded that the origin of the slow transformation in the pretransition reported by several methods [1–4] is caused by the slow formation or disappearance of the ripple structure observed in the equilibrated $P_{\beta'}$ phase.

Materials and Methods

DPPC (1,2-dipalmitoyl-*sn*-glycero-3-phosphorylcholine) was purchased from Sigma Chemical Co. The purity of the fatty acyl chains was determined to be over 98% by gas-liquid chromatography. Multilamellar liposomes were prepared as follows. The desired amount of phospholipid, determined by phosphorus assay according to Bartlett [6], was taken from a benzene solution and the solvent was evaporated first in a nitrogen stream and then by evacuation overnight. Distilled water and several glass beads were added and vortexed for 3 min with a Vortex-Genie mixer (Scientific Industry, Bohemia, NY) at 10°C above the phase transition temperature.

In a series of experiments for transition to $L_{\beta'}$ phase, the DPPC dispersion in small test tube was preincubated at 38°C for 2 h. Then, it was kept in a water-bath of 30°C for various periods of time (0.5, 7, 15, 40, 80 and 240 min). After the incubation at 30°C, a small volume of the dispersion was quickly pipetted into a specimen holder which had been prewarmed to 30°C. The sample was frozen immediately in liquid Freon 22, transferred to liquid nitrogen and then fractured in a freeze-etching device, HFZ-1 (Hitachi Co., Hitachi) at −110°C. The replica, prepared by platinum-carbon followed by carbon shadowing, was floated onto hypochlorous acid (HClO) and cleaned with

distilled water. The replica was then collected on 300 mesh grids and observed in a JEM-U electron microscope (JEOL, Tokyo).

In another series of experiments for transition to $P_{\beta'}$ phase, the DPPC dispersion was kept at 23°C for 12 h. Then, the sample was put into a water-bath at 35°C and kept for various times (0.5, 3 and 20 min). Freeze-fracture samples after the incubation were prepared following the procedure described for the transition to $L_{\beta'}$ phase.

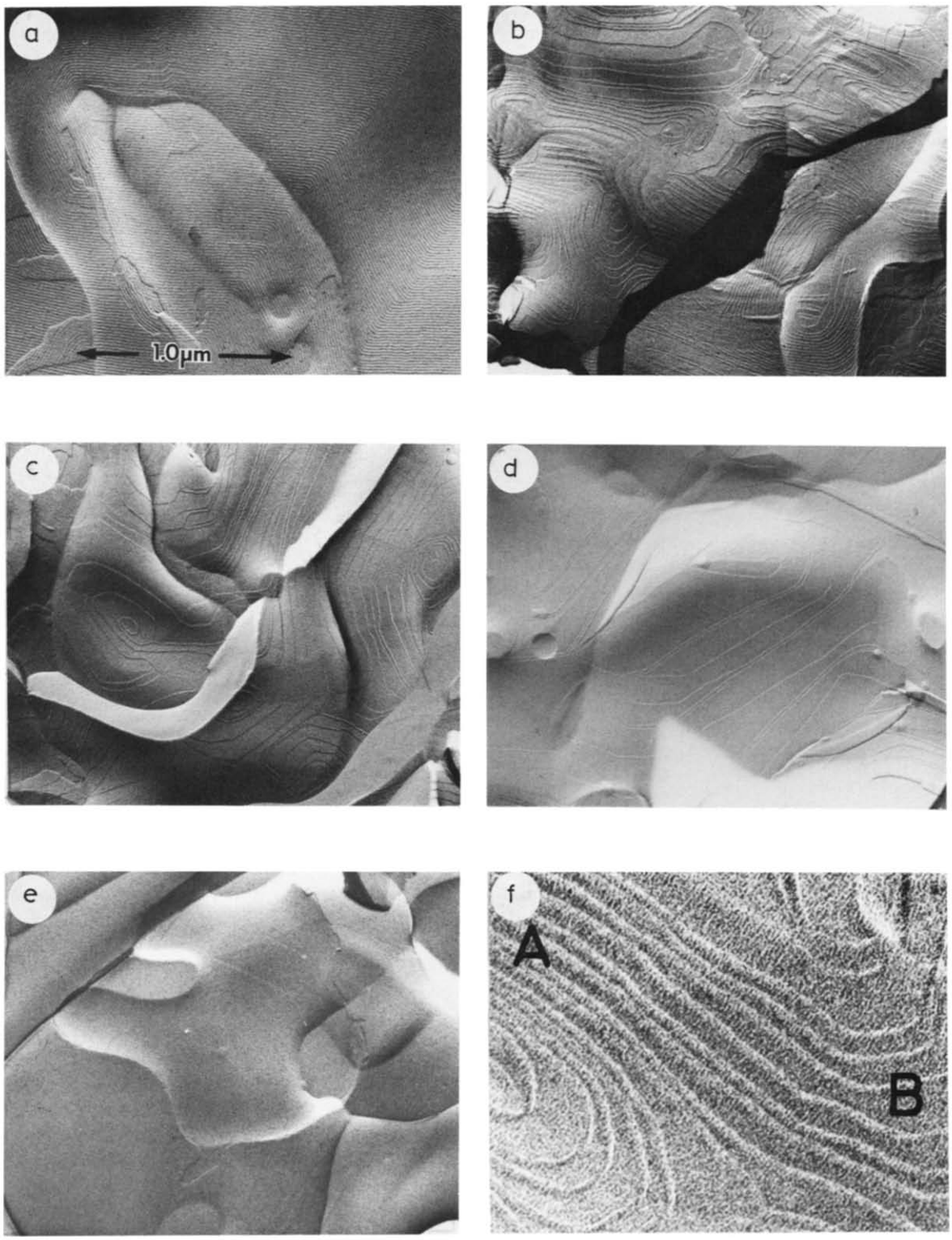
Results

X-ray diffraction is one of the most powerful methods for determining the phase of hydrated lipid dispersions. Under conditions of excess water, the L_α, $P_{\beta'}$ and $L_{\beta'}$ phases of DPPC liposomes are easily distinguished from each other by their X-ray diffraction patterns. Characteristic features of each phase also appears on the electron micrograph of freeze-fracture surface of lipid bilayer membranes [7–9]. The $P_{\beta'}$ exhibits the characteristic ripple structure as shown in Fig. 1a, while the $L_{\beta'}$ phase has an essentially smooth surface. The L_α phase also should exhibit a smooth surface. However, in most cases a 'jumbled structure' is observed because the nucleation of $P_{\beta'}$ phase is usually faster than the cooling rate in the quenching procedure. In the present study, freeze-fracture surfaces of DPPC liposomes were examined by electron microscopy under the same condition as was adopted in the previous study using the TEMPO spin probe. In the experiment of relaxation from $P_{\beta'}$ to $L_{\beta'}$, DPPC liposomes equilibrated to 38°C were quickly cooled to 30°C, then the freeze-fracture surfaces were examined after various incubation times (0.5, 7, 15, 40, 80 and 240 min). Typical patterns in the relaxation process are shown in Fig. 1. At 0.5 min after cooling to 30°C, the regular banded pattern observed in $P_{\beta'}$ phase (Fig. 1a) is expanded irregularly, and the ridges of ripple structure are no longer parallel to each other (Fig. 1b). Consequently, the density of ripple decreases on the surface. The pattern of the ripple shown in Fig. 1c is almost the same as the pattern in Fig. 1b except for the interval between the neighboring ripple ridges; it becomes wider in Fig. 1c. In the next micrograph at 80 min, the ripples become obscure without the further expansion of the ripple interval, and they are no longer continuous lines (Fig. 1d). At 240 min after the temperature shift, all ripple structures disappear in the fractured surface, and the transition to $L_{\beta'}$ phase is completed (Fig. 1e).

Fig. 1f shows the magnified micrograph of the lower part of Fig. 1b. This micrograph reveals the origin of the expansion of the ripple interval. If a ripple disappears after the temperature jump, the interval between the remaining two ripples should become twice the initial interval. However, the interval between ripples is the same as that of the $P_{\beta'}$ phase in region A and it becomes gradually wider toward regions B. A similar phenomenon is also observed in Fig. 1c. This fact suggests that the increase of the ripple interval is caused by the lateral movement of ripples rather than by the disappearance of an intermediate ripple. Moreover, almost all ripples make continuous lines and a disconnected ripple line is rarely observed in Figs. 1b and 1c. These results also support that the ripple pattern in Figs. 1b and 1c originates from the expansion of the interval between the ripples rather than from the vanishing of intermediate ripples.

As shown in electron micrographs of Fig. 1, the change of ripple interval reflects the process of relaxation. So the density of ripples was estimated by counting the number of ripples crossing a 0.5 μm bar oriented perpendicularly at typical points of each electron micrograph. Fig. 2 shows the time-course of ripple density in the freeze-fracture surfaces of DPPC liposomes shifted from 38°C to 30°C. The solid line is the single relaxation function, $\exp(-t/t_1)$, calculated from the fast relaxation time obtained in the previous ESR measurement of Ref. 4 ($t_1 = 2$ min at 30°C). Considering the error of the quenching temperature in the freeze-fracturing method (± 1°C), the time dependence of ripple density decrease is very compatible with the fast relaxation process observed in the previous study using TEMPO spin probes. This result suggests that there is a close relationship between the fast relaxation process in the ESR measurement and the decrease of ripple density in the freeze-fracture surface.

The relaxation process was also examined in the opposite direction of temperature change; from $L_{\beta'}$ phase to $P_{\beta'}$ phase. DPPC liposomes were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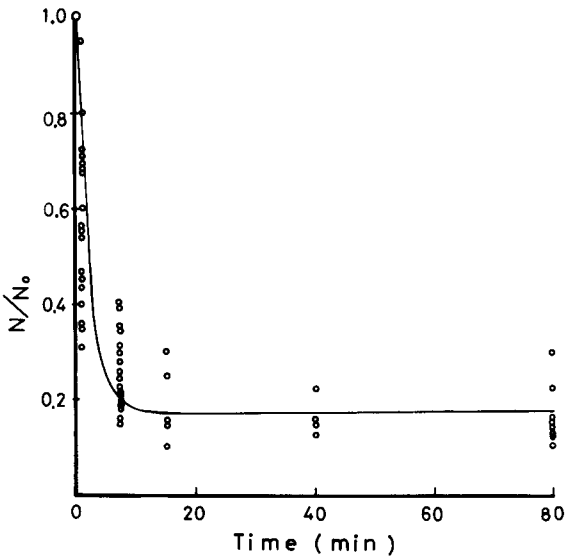

Fig. 2. The time-course of the normalized ripple density. N is the number of ripple crossing a test bar of 0.5 μm oriented perpendicular to the ripple ridge lines. Estimations were carried out at several points in each electron micrograph obtained in the same experimental condition, N_0 is the value obtained from 38°C. The solid line is the single relaxation curve of $\exp(-t(\text{min})/2)$ obtained in the previous study using TEMPO spin probe.

kept at 23°C for 12 h to be equilibrated to the $L_{\beta'}$ phase. The sample was quickly transferred to 35°C, and quenched from 35°C after different incubation times: 0.5, 3 and 20 min. Electron micrographs of the freeze-fracture surfaces of these samples are shown in Fig. 3. Some differences are evident in this process compared to the reverse process from $P_{\beta'}$ to $L_{\beta'}$. The relaxation process of $L_{\beta'}$ to $P_{\beta'}$ is 10-fold faster than that of $P_{\beta'}$ to $L_{\beta'}$. Bundles of regular ripple structures already appear in the freeze-fracture surface at 0.5 min after the temperature shift (Fig. 3a). Ripples make continuous parallel lines with an interval of about 13 nm between the ripple ridges, which is the maximum density of the ripples in the $P_{\beta'}$ phase. The following is one plausible mechanism of the creation of ripple structure. A set of ripple structures that has a 13 nm repeat interval appears at an early stage, and then these regions spread out all over the membranes within several minutes.

Discussion

In a previous study [4] we examined the relaxation process of the phase transition of DPPC liposomes around the pretransition temperature using TEMPO spin probe. In the present study the relaxation process of the phase change in DPPC liposomes was investigated by freeze-fracture electron microscopy. In the transition of $P_{\beta'}$ phase to $L_{\beta'}$ phase, two different processes were observed in the freeze-fracture surface of DPPC liposomes. The relaxation times of the fast process obtained from the change of ripple structures are in good agreement with those determined in the previous study using TEMPO spin probe [4]. The present study suggests that the fast process is due to the expansion of the intervals between the ripple ridges. The increase of the ripple interval seems to be caused by the movement of the ripples toward a vanishing region. However, we could not directly detect the direction of the movement and the place where the ripples disappeared after the movement. One possible mechanism is that the ripples move toward the center of the concentric configuration of ripple structure and vanish in the center of the concentric circles (because the movement toward the center results in the decrease of the ripple length or the reduction of ripple lines). Following this fast process, the dispersed ripples disappear gradually. This step was observed in the fractured surface of DPPC liposomes at 80 min after the temperature shift. The ripples disappear completely at 240 min after the temperature shift. This disappearance process of individual ripple seems to be related to the slow relaxation process observed in the previous ESR measurement (t_2 = 43 min at 30°C).

In the temperature shift from $L_{\beta'}$ phase to $P_{\beta'}$

Fig. 1. The freeze-fracture electron micrographs of DPPC multilamellar liposomes. The liposomes were quenched (a) from 38°C, or (b)–(f) from 30°C after temperature shifts from 38°C to 30°C. The holding times at 30°C were (b) 0.5 min, (c) 7 min, (d) 80 min and (e) 240 min. The direction of shadowing is from the bottom to the top of each micrograph. (f) Magnified micrograph of the lower part in Fig. 1b.

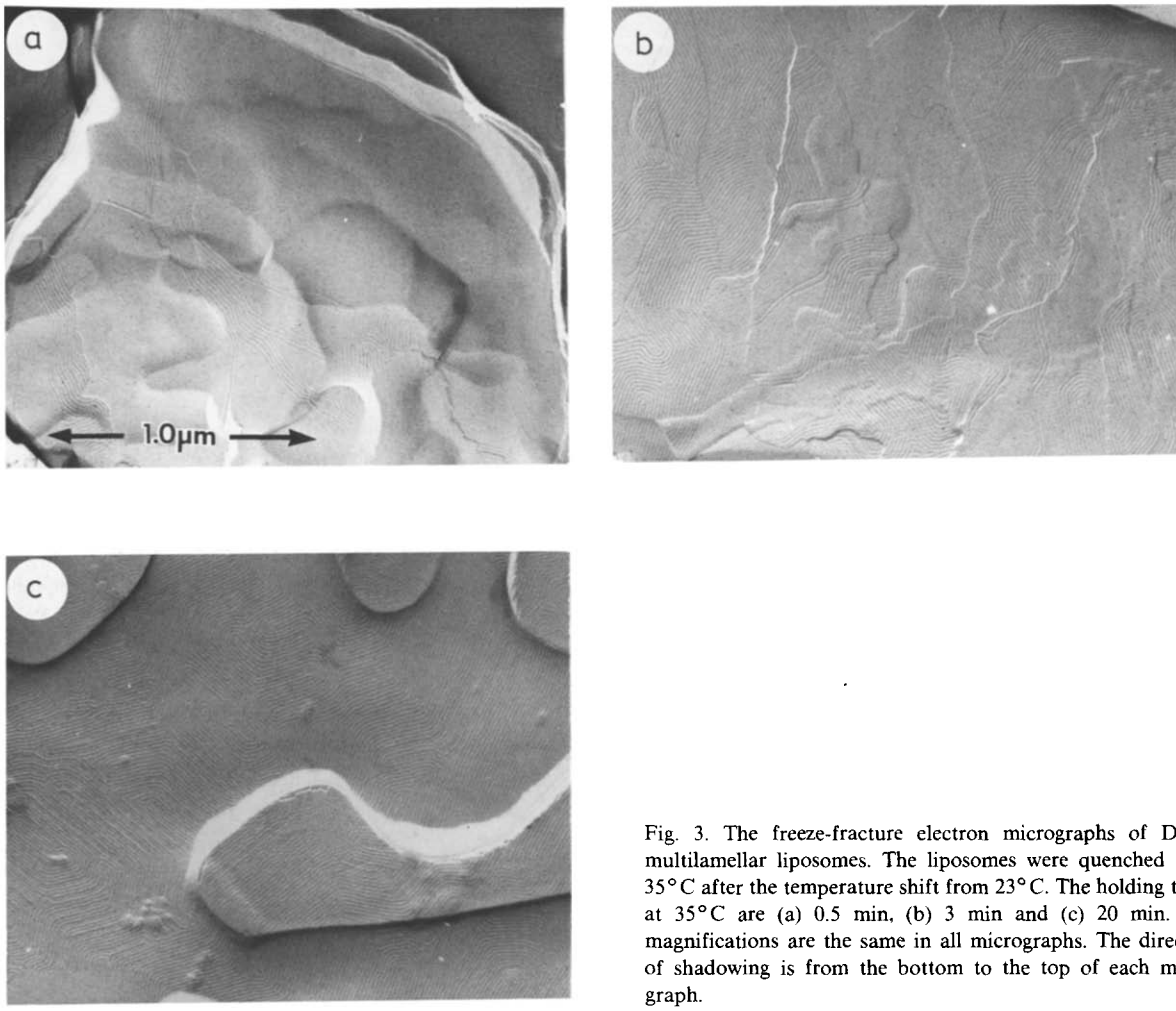

Fig. 3. The freeze-fracture electron micrographs of DPPC multilamellar liposomes. The liposomes were quenched from 35°C after the temperature shift from 23°C. The holding times at 35°C are (a) 0.5 min, (b) 3 min and (c) 20 min. The magnifications are the same in all micrographs. The direction of shadowing is from the bottom to the top of each micrograph.

phase, a set of ripple structures that has a 13 nm repeat interval appears at an early stage, and then these regions spread out all over the membranes within several minutes. The fraction of membrane showing ripple structure is, approximately, 20% at 0.5 min, 75% at 3 min and 100% at 20 min of the whole fractured surface as shown in Fig. 3. The rough estimation gives a single relaxation process with a relaxation time of about 130 s. This value is comparable to the slow relaxation process of the ESR measurement; $t_2 = 75$ s at 35.0°C and $t_2 = 170$ s at 34.4°C.

In the previous ESR study [4] the process of the relaxation was found to depend on the magnitude of the temperature shift. Now we are proceeding a series of experiments using freeze-fracture electron microscopy after temperature shifts of various magnitudes.

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