

Two-Photon Fluorescence Microscopy Studies of Bipolar Tetraether Giant Liposomes from Thermoacidophilic Archaeobacteria *Sulfolobus acidocaldarius*

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ABSTRACT The effects of temperature and pH on Laurdan (6-lauroyl-2-(dimethylamino)naphthalene) fluorescence intensity images of giant unilamellar vesicles (GUVs) (~20–150 μm in diameter) composed of the polar lipid fraction E (PLFE) from the thermoacidophilic archaeobacteria *Sulfolobus acidocaldarius* have been studied using two-photon excitation. PLFE GUVs made by the electroformation method were stable and well suited for microscopy studies. The generalized polarization (GP) of Laurdan fluorescence in the center cross section of the vesicles has been determined as a function of temperature at pH 7.23 and pH 2.68. At all of the temperatures and pHs examined, the GP values are low (below or close to 0), and the GP histograms show a broad distribution width (> 0.3). When excited with light polarized in the y direction, Laurdan fluorescence in the center cross section of the PLFE GUVs exhibits a photoselection effect showing much higher intensities in the x direction of the vesicles, a result opposite that previously obtained on monopolar diester phospholipids. This result indicates that the chromophore of Laurdan in PLFE GUVs is aligned parallel to the membrane surface. The x direction photoselection effect and the low GP values lead us to further propose that the Laurdan chromophore resides in the polar headgroup region of the PLFE liposomes, while the lauroyl tail inserts into the hydrocarbon core of the membrane. This unusual L-shaped disposition is presumably caused by the unique lipid structures and by the rigid and tight membrane packing in PLFE liposomes. The GP exhibited, at both pH values, a small but abrupt decrease near 50°C, suggesting a conformational change in the polar headgroups of PLFE. This transition temperature fully agrees with the d -spacing data recently measured by small-angle x-ray diffraction and with the pyrene-labeled phosphatidylcholine and perylene fluorescence data previously obtained from PLFE multilamellar vesicles. Interestingly, the two-photon Laurdan fluorescence images showed snowflake-like lipid domains in PLFE GUVs at pH 7.23 and low temperatures ($< 20^\circ\text{C}$ in the cooling scan and $< 24^\circ\text{C}$ in the heating scan). These domains, attributable to lipid lateral separation, were stable and laterally immobile at low temperatures ($< 23^\circ\text{C}$), again suggesting tight membrane packing in the PLFE GUVs.

INTRODUCTION

The thermoacidophilic archaeobacterium *Sulfolobus acidocaldarius* lives at high temperatures (normally 65–80°C) and in acidic environments (pH 2–3), while its intracellular compartment is near neutral. The ability of the organism to sustain such harsh growth conditions has something to do with the properties of its plasma membrane lipids. The major component of the plasma membrane of *S. acidocaldarius* is bipolar tetraether lipids (~90% of the total lipids) (Langworthy and Pond, 1986; De Rosa et al., 1986; Kates, 1992). Among them, the polar lipid fraction E (PLFE) is the main constituent (Lo and Chang, 1990). Approximately 90% of PLFE lipids are a glycerol dialkylnonitol tetraether (GDNT) containing phosphatidylmyoinositol on one end and β -glucose on the other (Fig. 1). About 10% are glycerol dialkylglycerol tetraether (GDGT) with phosphatidylmyoinositol attached to one glycerol and β -D-galactosyl-D-glucose attached to the other (Fig. 1). Both GDGT and GDNT

consist of a pair of 40-carbon phytanyl hydrocarbon chains. Each of the biphytanyl chains contains up to four cyclopentane rings, and the number of these rings increases with increasing growth temperature (De Rosa and Gambacorta, 1988).

In aqueous solutions PLFE lipids extracted from *S. acidocaldarius* form stable multilamellar liposomes with vortexing and unilamellar vesicles (diameters ~60–800 nm) with extrusion (Lo and Chang, 1990; Elferink et al., 1992; Komatsu and Chong, 1998). These liposomes exhibited no or only broad and small-magnitude endothermic phase transitions in the temperature range of 10–70°C (personal communications with E. Chang). Electron microscopy showed that in PLFE liposomes the lipids span the entire lamellar structure, forming a monomolecular membrane (Elferink et al., 1992). Compared to liposomes prepared from “normal” diester lipids, PLFE liposomes exhibit high thermal stability in terms of the unusually low rates of proton permeation (Elferink et al., 1994; Komatsu and Chong, 1998) and carboxyfluorescein leakage (Elferink et al., 1994; Chang, 1994; Komatsu and Chong, 1998). The thermal stability has been attributed to the rigid and tight membrane packing in PLFE liposomes (Komatsu and Chong, 1998). The tightness of membrane packing increases with increasing the number of cyclopentane rings in the phytanyl chain of PLFE lipids (Gabriel and Chong, 2000).

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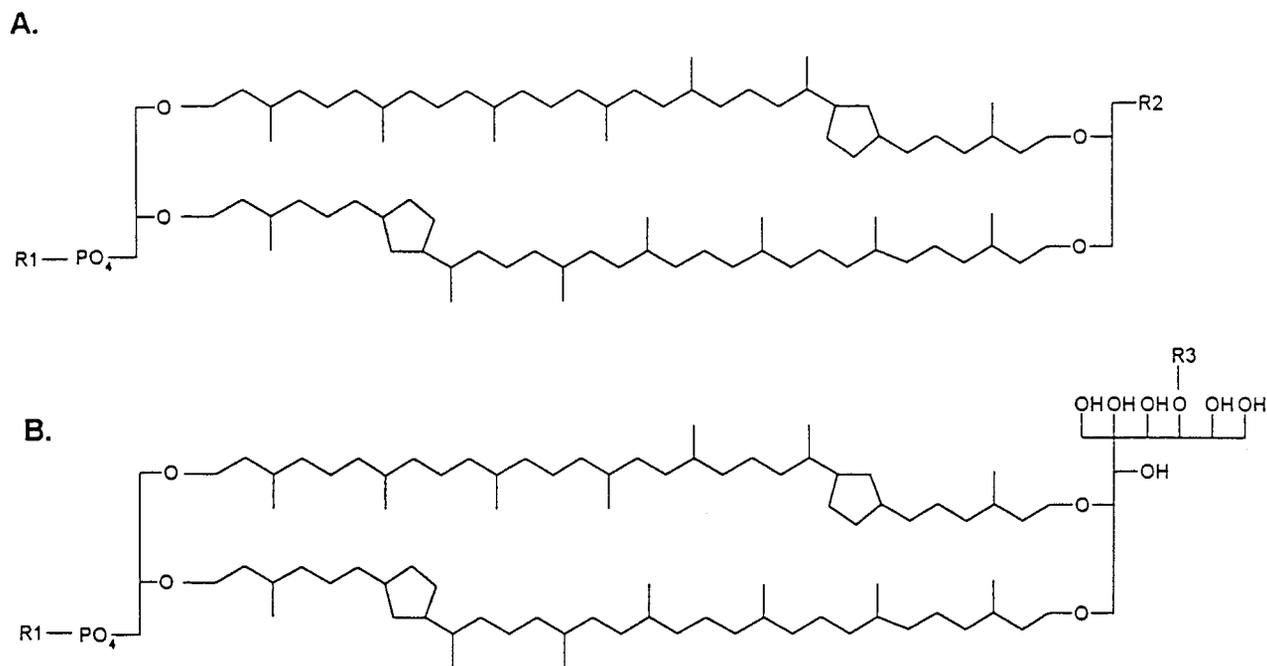


FIGURE 1 Structures of PLFE lipids. (A) Glycerol dialkylglycerol tetraether (GDGT). (B) Glycerol dialkylnonitol tetraether (GDNT). R1 = inositol; R2 = β -D-glucopyranose; R3 = β -D-galactosyl- β -D-glucopyranose. The number of cyclopentane rings can vary from 0 to 4 in each phytanyl chain (De Rosa and Gambacorta, 1988 (adopted from Lo and Chang, 1990).

While PLFE lipids provide archaeobacteria with a rigid and tight barrier between the intracellular and extracellular environments, they must also provide archaeobacterial membranes with some “fluidity” to exhibit functionality (In’t Veld et al., 1992; Elferink et al., 1993). To understand archaeobacterial “membrane fluidity,” we have previously examined the lateral and rotational diffusions of membrane probes in PLFE multilamellar vesicles (MLVs). The lateral mobility of a pyrene-labeled phosphatidylcholine in PLFE MLVs was found to be highly restricted and only became significant at temperatures higher than 48°C (Kao et al., 1992). The rotational rate of perylene in PLFE MLVs also undergoes an abrupt increase at \sim 48°C (Khan and Chong, 2000). These changes in dynamic motions appear to be related to the structural changes, as the *d*-spacing measured by small-angle x-ray diffraction also exhibited an abrupt increase above \sim 50°C (Zein et al., unpublished results). Despite these efforts, the molecular details of membrane packing and dynamics in the water-membrane interfacial region (including the glycerol backbone and the polar headgroups) of PLFE liposomes remain very much unexplored. Furthermore, it is uncertain whether the MLV studies mentioned above contain any artifacts due to vesicle aggregation and/or the inhomogeneous distribution in vesicle size and shape.

Laurdan (6-lauroyl-2-(dimethylamino)naphthalene), like its parent compound Prodan (6-propionyl-2-(dimethylamino)naphthalene) (Weber and Farris, 1979), is a polarity-sensitive membrane probe, with its chromophore located at

or near the membrane-water interfacial region (Parasassi et al., 1991; Chong and Wong, 1993; Zeng et al., 1995; Bagatolli et al., 1998). Combining the sectioning effect of the two-photon fluorescence microscope and the well-characterized fluorescent properties of Laurdan, we recently reported shape hysteresis at the phase transition temperature in single-component giant unilamellar vesicles (GUVs) composed of monopolar diester phospholipids. We also reported fluorescence images of solid and fluid lipid domains in GUVs composed of binary mixtures of monopolar diester phospholipids (Bagatolli and Gratton, 2000).

In this work, we have examined the effects of temperature and pH on Laurdan fluorescence intensity images of GUVs composed of PLFE lipids from *S. acidocaldarius*, using two-photon excitation microscopy. Our strategy is based on monitoring single PLFE GUVs at different temperatures and pHs. The unique properties of unsupported GUVs allow us to make new observations about the shape and morphology of liposomes derived from archaeobacterial membranes in an environment similar to that found in cells.

MATERIALS AND METHODS

Materials

S. acidocaldarius cells (strain DSM639; ATCC, Rockville, MD) were grown aerobically and heterotrophically at 69–70°C, pH 2.5–3.0. PLFE lipids were isolated from *S. acidocaldarius* dry cells by soxhlet extraction with chloroform:methanol (1:1, v/v) for 48 h, as previously described (Lo

and Chang, 1990). In brief, the crude lipids were fractionated by reversed-phase column chromatography, using C-18 PrepSep columns (Fisher Scientific, Fair Lawn, NJ), eluted first with methanol:water (1:1, v/v) (filtrate A) and then with chloroform:methanol:water (0.8:2:0.8, v/v) (filtrate B). Filtrate B was further separated by thin-layer chromatography (TLC) (PLK5 silica gel 150A; Whatman, NJ), using a mobile phase of chloroform:methanol:water (65:25:4, v/v). The PLFE fraction ($R_f \approx 0.2$) was scraped from silica TLC plates and eluted with chloroform:methanol:water (1:2:0.8, v/v). Finally, PLFE was purified by cold methanol precipitation two to three times. Laurdan (Molecular Probes, Eugene, OR) was dissolved in dimethyl sulfoxide (DMSO) (0.45 nmol/ μ l) and stored in the dark at 4°C before use.

MATERIALS AND METHODS

Vesicle preparation

GUVs of PLFE were prepared at 65°C by the electroformation method (Dimitrov and Angelova, 1987; Angelova and Dimitrov, 1988; Angelova et al., 1992), using a home-built chamber containing two parallel platinum wires (Bagatolli and Gratton, 1999). Twenty-one microliters of 0.23 mg/mL PLFE in chloroform:methanol:water (65:25:10) was applied to each wire under a stream of nitrogen. The lipids on the wires were further dried under vacuum (10 millitorr) for ~1–2 h. Five milliliters of hot (~65°C) buffer (either 1 mM sodium pyrophosphate/citrate at pH 7.23 or 1 mM citrate at pH 2.86) was loaded into the chamber, and the solution was ramped to 65°C. The platinum wires were then connected to a function generator (model 3325; Hewlett-Packard, Santa Clara, CA). To make PLFE GUVs at pH 7.23, an AC field 1 V in amplitude and 10 Hz in frequency was applied to the platinum wires for ~90 min. To generate PLFE GUVs at pH 2.86, a higher voltage (2.5 V in amplitude) was employed. The AC signal was disconnected before two-photon excitation of Laurdan in PLFE GUVs.

Two-photon fluorescence measurements of Laurdan GP

Two-photon excitation microscopy of Laurdan fluorescence in PLFE GUVs was performed as previously described (Yu et al., 1996; Parasassi et al., 1997; Bagatolli and Gratton, 1999, 2000). In brief, an Axiovert 35 inverted microscope (Zeiss) was used with a 20 \times LD-Achroplan (0.4 N.A., air) objective (Zeiss). A titanium-sapphire laser (Mira 900; Coherent, Palo Alto, CA) pumped by a frequency-doubled Nd-vanadate laser (Verdi; Coherent) was used as the light source with a 780-nm excitation wavelength. This two-photon excitation is equivalent to one-photon excitation at 390 nm. The laser polarized in the y direction was guided by a galvanometer-driven x - y scanner (Cambridge Technology, Watertown, MA) to achieve beam scanning in both the x and y directions. A frequency synthesizer (Hewlett-Packard) controlled the scanning rate, and a frame rate of 20 s was used to acquire the images (256 \times 256 pixels). The pixel size corresponded to 0.52 μ m. The sample received a laser power of ~10 mW.

For fluorescence labeling, ~6 μ l of 0.0045 nmol/ μ l Laurdan in DMSO was added to a solution of 5 ml of PLFE GUVs in the electroformation chamber at 65°C. This yields a probe-to-PLFE ratio of ~1/170, which is equivalent to ~1/340 in the case of monopolar phospholipids. The amount of DMSO added has previously been shown not to perturb membrane structure to any significant extent (Bagatolli and Gratton, 1999, 2000). The center cross section of the vesicles was visualized through the microscope, and the excitation generalized polarization ($GP_{ex} = (I_B - I_R)/(I_B + I_R)$; Parasassi et al., 1990, 1991) was measured as a function of temperature. Here I_B and I_R are the fluorescence intensities at the blue and red edges of the emission spectrum, respectively. I_B and I_R were measured with two band-pass filters (Ealing Electro-optics, Holliston, MA) with 46-nm bandwidth and transmittance centered at 446 nm (blue filter) and 499 nm (red

filter), respectively. A photomultiplier (R5600-P; Hamamatsu, Bridgewater, NJ) was used for fluorescence detection in the photon counting mode. The temperature of the GUVs was measured by using a thermocouple affixed to the platinum wires. In addition to the center cross section, the top and bottom cross sections were also visualized via Laurdan fluorescence intensity for possible domain formation in the membrane.

RESULTS

Formation of PLFE GUVs

Using a charge-coupled device camera, we have observed, in real time, the formation of PLFE GUVs from the lipid film previously deposited on the platinum wires. At pH 7.23, once an AC field of 1 V in amplitude and 10 Hz in frequency was applied, the lipids on the platinum wires began to vibrate (an electroosmotic effect; Dimitrov and Angelova, 1988), and, in a few minutes, GUVs were formed. The yield of GUVs was comparable to that observed in monopolar diester phospholipids (95%) (Bagatolli and Gratton, 1999, 2000). While the majority of the vesicles had a diameter of ~20–50 μ m, some were of very large sizes (~150 μ m in diameter). The PLFE GUVs maintained a spherical shape over a wide range of temperatures (65–10°C) during the time course of the experiment.

GUVs at pH 2.86 can be generated using an AC field at a higher voltage (2.5 V; 10 Hz); however, the vesicle yield was significantly lower than that at pH 7.23. The need for a higher voltage and the low vesicle yield are expected (Dimitrov and Angelova, 1987), because PLFE lipid films become less negatively charged and thus more tightly packed at pH 2.86. Despite the low yield, PLFE GUVs at pH 2.86 were spherical and stable. It is worth mentioning that, when a lower AC field (1 V) was applied to PLFE lipids at pH 2.86, many small vesicles were formed in conjunction with few GUVs. The small vesicles quickly moved into the bulk solution, and some of them attached to the top of the GUVs, interfering with fluorescence imaging.

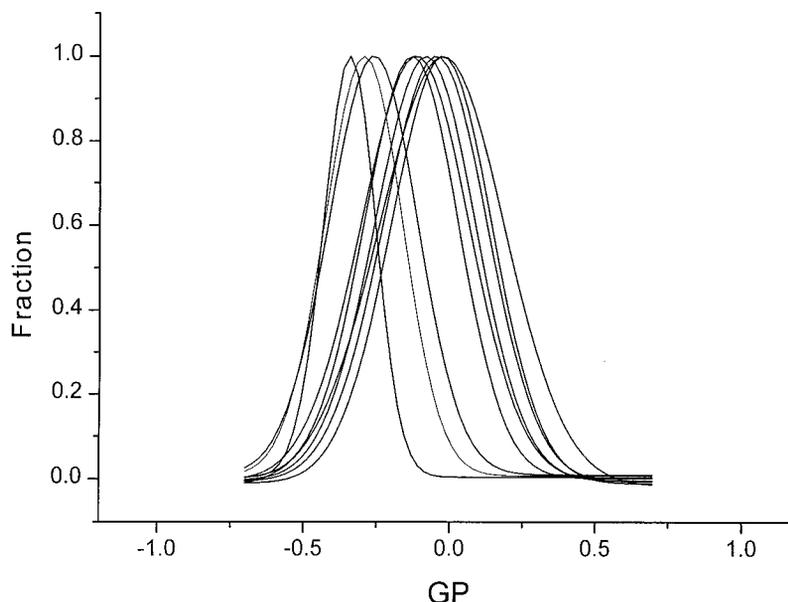
With the appropriate AC signals, bipolar tetraether PLFE GUVs can be generated by the electroformation method at both pH 7.23 and pH 2.86. At these pHs, PLFE liposomes are negatively charged. In comparison, the negatively charged monopolar diester lipid phosphatidylserine was reported not to form GUVs in the presence of an electric field (Dimitrov and Angelova, 1987).

Effect of temperature on Laurdan GP in PLFE GUVs

Two-photon fluorescence images of the PLFE GUVs (>20 μ m in diameter) adsorbed to the platinum wires were recorded at various temperatures. At each temperature, the center cross section as well as the top and bottom surfaces of the target vesicle were scanned.

The effect of temperature on Laurdan's GP in the center cross section of PLFE GUVs at pH 7.23 was determined in

FIGURE 2 Effect of temperature on the distribution of Laurdan GP from the images of the center cross section of PLFE GUVs at pH 7.23. Temperature (from left to right): 66.0, 57.0, 52.0, 50.3, 49.2, 46.2, 39.6, 30.8, and 12.0°C.

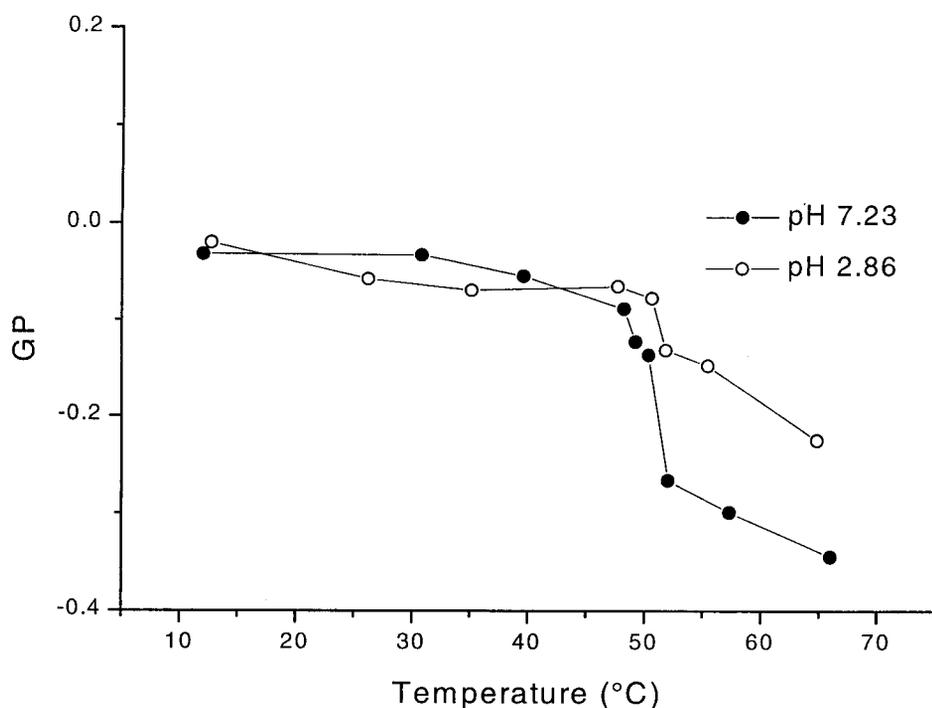


a cooling mode ($\sim 0.18^\circ/\text{min}$) from 66.0 to 12.0°C (Figs. 2 and 3). At each temperature, the GP histogram is fit to a Gaussian distribution. The normalized Gaussian distributions of Laurdan's GP at various temperatures are presented in Fig. 2. The maximum GP values (average GP) in the histograms are plotted against temperature (Fig. 3). The plot shows that initially there is a small but steady increase in the average GP from 66.0 to 52.0°C, which is followed by an abrupt increase at $\sim 50^\circ\text{C}$. Thereafter, the average GP in-

creases slightly with increasing temperature. At all of the temperatures examined, the GP distributions in PLFE GUVs are wide (>0.3) and the average GP values are below 0, similar to the cases in liquid-crystalline monopolar diacylphosphatidylcholine GUVs (Parasassi et al., 1997; Bagatolli and Gratton, 2000).

The effect of temperature on Laurdan's GP in the center cross section of PLFE GUVs at pH 2.86 was also determined, in a cooling mode ($\sim 0.15^\circ/\text{min}$) from 64.8 to 12.7°C

FIGURE 3 The temperature dependence of Laurdan GP from the images of the center cross section of PLFE GUVs at pH 7.23 (●) and pH 2.86 (○).



(Figs. 3 and 4). As in the case of pH 7.23, the average GP values at pH 2.86 are low (Fig. 3). The average GP at pH 2.86 also exhibits an abrupt change at $\sim 50^\circ\text{C}$; however, the change is less pronounced, as compared to the case of pH 7.23 (Fig. 3). Below 46°C , the average GP values appear to be independent of pH. In contrast, above 52°C , the average GP values at pH 2.86 are greater than at pH 7.23 (Fig. 3).

The GP histogram and the average GP in the center cross section of PLFE GUVs at a given temperature and pH are reproducible from different vesicles in the same electroformation chamber and from separate vesicle preparations.

Laurdan dipole orientation in PLFE GUVs

Fig. 5 shows that when the excitation light is polarized in the y direction, Laurdan's fluorescence intensity in the center cross section of the vesicle is much brighter in the x direction than in the y direction. This photoselection effect holds true for both pH 7.23 and pH 2.86 in PLFE GUVs at all of the temperatures examined. In contrast, under the same excitation conditions, the two-photon images of Laurdan fluorescence in monopolar diester phospholipid GUVs show a preferred photoselection in the y direction (Bagatolli and Gratton, 2000; Fig. 5).

Domain formation

In a cooling scan, we noticed lipid domain coexistence in PLFE GUVs at pH 7.23 below $\sim 20^\circ\text{C}$. The left panel of Fig. 6 shows Laurdan's fluorescence intensity image in the center cross section of the vesicle at 12.4°C . At this low temperature, Laurdan's fluorescence intensity continues to show the photoselection effect in the x direction; however,

discontinuity of the intensity brightness appears in the vesicle border, indicating that Laurdan is segregated from these lipid domains (Bagatolli and Gratton, 2000). The center panel of Fig. 6 shows the cross section toward the top of the vesicle at 11.5°C , from which snowflake-like domains are visualized. At the very top of the same vesicle (Fig. 6, right), the snowflake-like domains are even more clearly observable. This phenomenon occurs in virtually all of the vesicles in the chamber at low temperatures ($< \sim 20^\circ\text{C}$) and is reproducible from separate vesicle preparations. These domains appear to be stable with time at a given temperature (data not shown).

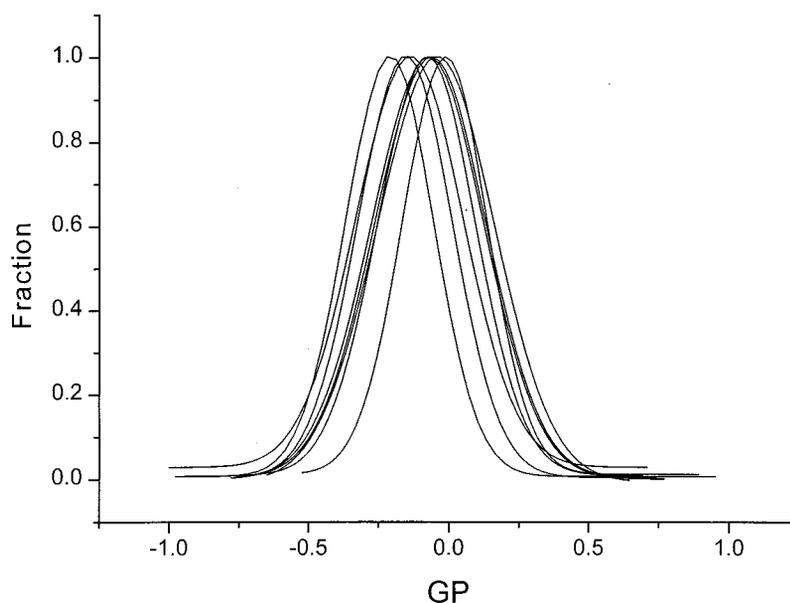
The temperature-induced snowflake-like domains are reversible in the heating scan. Fig. 7 shows a series of two-photon Laurdan fluorescence intensity images obtained from the top of the vesicle at different temperatures during the heating scan. At 12.8 – 23.2°C , the snowflake-like domains were immobile within the vesicle. When elevated to 24°C , these domains began to show lateral mobility. The domains shrunk between 24 and 28°C and eventually disappeared at 29.2°C . Apparently, the domain formation shows temperature hysteresis between the heating and cooling scans.

At pH 2.86, domain coexistence also occurs in PLFE GUVs at low temperatures (Fig. 8). However, in this case, the lipid domains show irregular shape, in contrast to the case at pH 7.23.

DISCUSSION

The use of Laurdan fluorescence in membrane studies has been increasing in recent years, mainly because of the high sensitivity of Laurdan's emission spectrum to environmen-

FIGURE 4 Effect of temperature on the distribution of Laurdan GP from the images of the center cross section of PLFE GUVs at pH 2.86. Temperature (from left to right): 64.8, 55.4, 51.8, 50.6, 47.4, 35.1, 26.2, and 12.7°C .



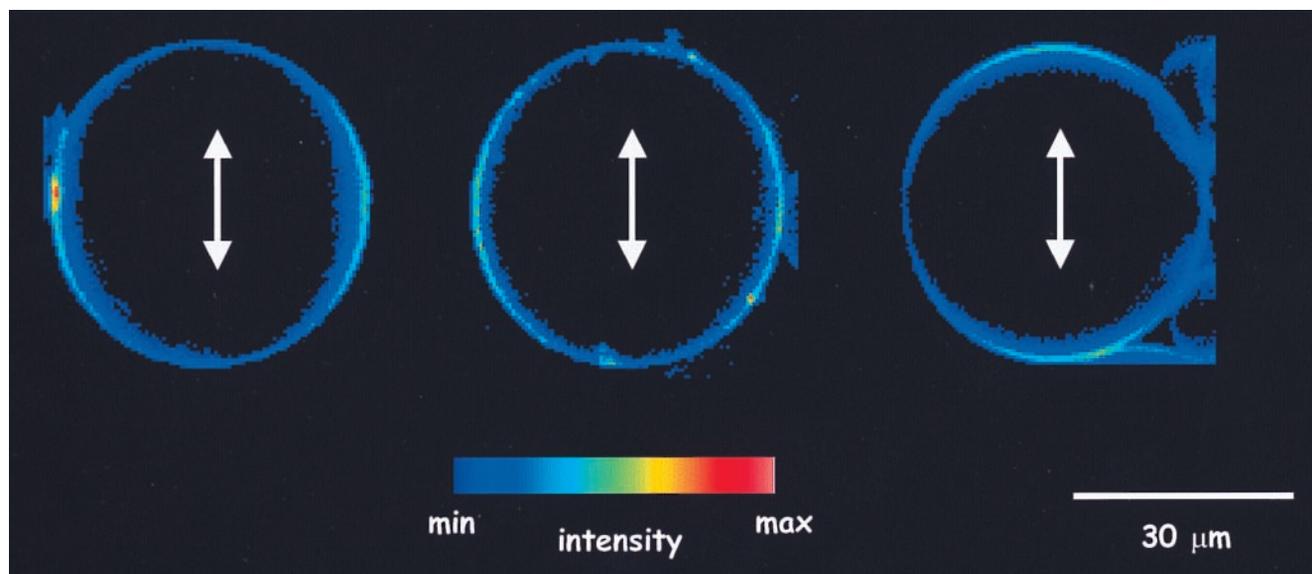


FIGURE 5 Comparison of the photoselection effects between the Laurdan fluorescence intensity images obtained from bipolar tetraether PLFE GUVs (*left*: at pH 7.23; *middle*: pH 2.86) and that from monopolar diester phospholipid GUVs (*right*: for DPPC). The white arrows indicate the polarization orientation of the excitation beam.

tal polarity and the physical state of the membrane (reviewed in Parasassi and Gratton, 1995; Parasassi et al., 1998). However, interpretation of Laurdan fluorescence data requires knowledge of chromophore location and the

orientation of the emission dipole. Infrared and fluorescence studies on monopolar diester phospholipid bilayers indicated that the chromophore of Laurdan is embedded in the hydrocarbon region near the glycerol backbone (Parasassi et

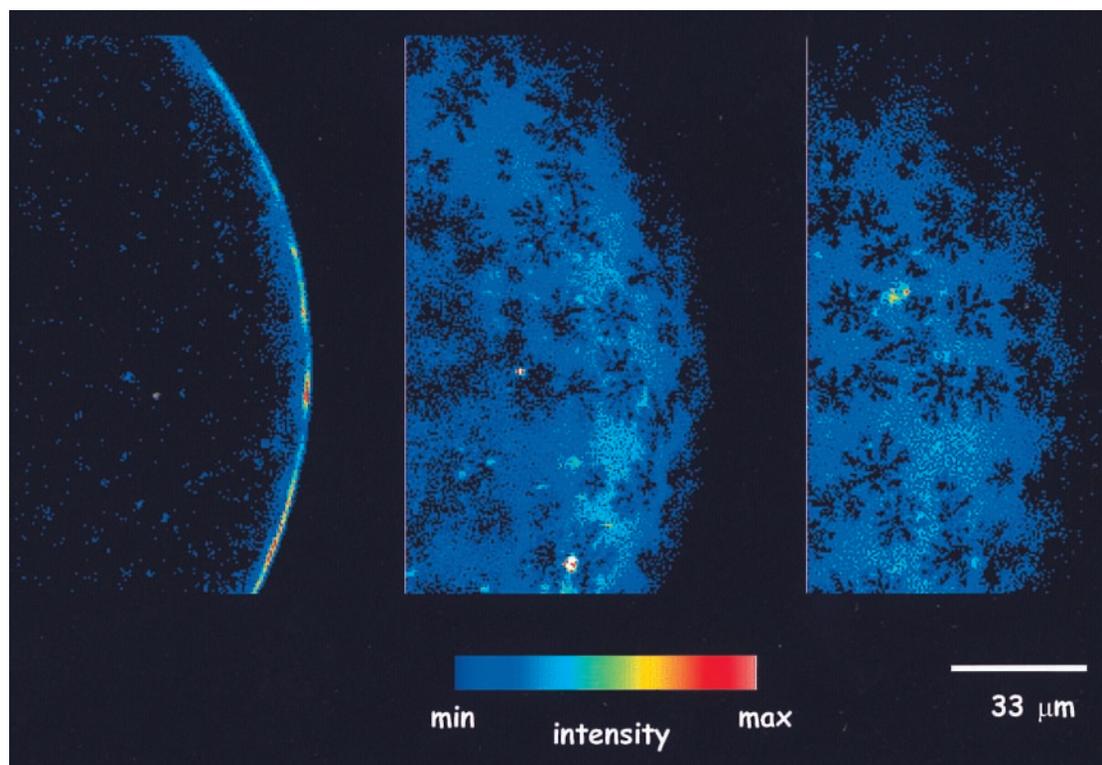


FIGURE 6 Patterns of the snowflake-like domains in PLFE GUVs at pH 7.23 and low temperatures. (*Left*) The Laurdan fluorescence intensity image in the center cross section of the vesicle. (*Middle*) The image toward the top of the same vesicle. (*Right*) The image at the very top of the same vesicle.

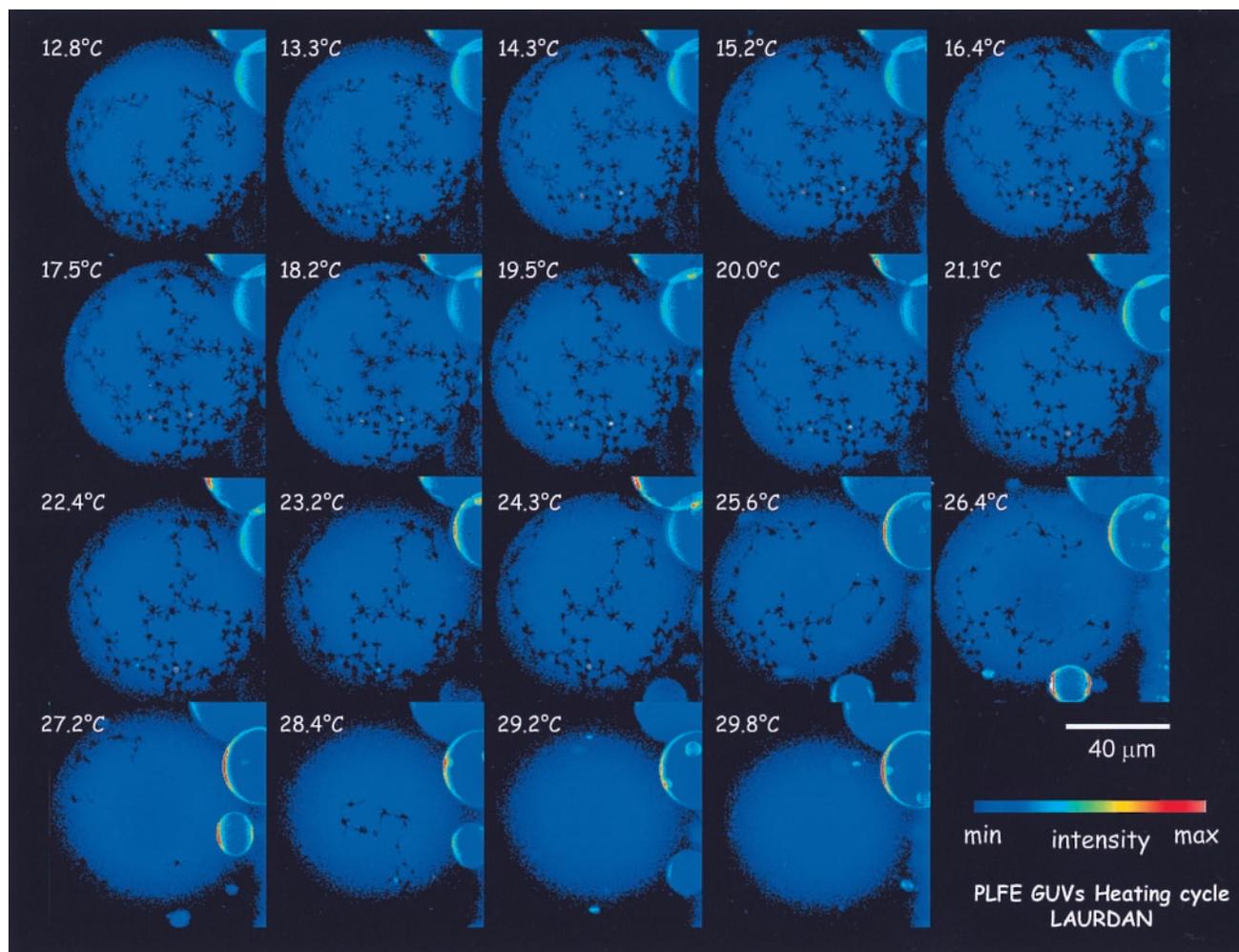


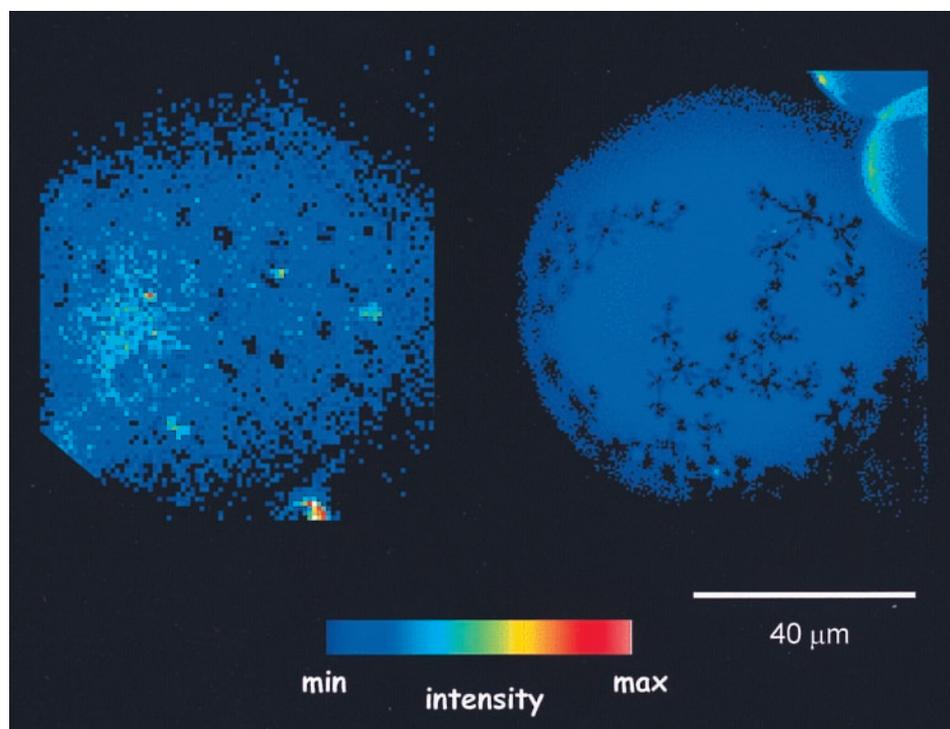
FIGURE 7 Two-photon Laurdan fluorescence intensity images in PLFE GUVs at pH 7.23 as a function of temperature in a heating mode. All of the images were obtained from the top of the same vesicle.

al., 1991; Chong and Wong, 1993; Zeng and Chong, 1995; Bagatolli et al., 1999). Recent two-photon microscopy studies showed that the emission dipole of Laurdan is aligned perpendicular to the membrane surface of monopolar diester phospholipids (Bagatolli and Gratton, 1999, 2000).

The current study, however, presents a totally different case for the location of Laurdan chromophore and for the orientation of Laurdan emission dipole in the membrane. The images shown in Fig. 5 clearly indicate that the emission dipole of Laurdan in bipolar tetraether PLFE GUVs is mainly aligned parallel to the membrane surface, just the opposite of the situation in monopolar diester phospholipid GUVs. Three possible configurations can be proposed for Laurdan in PLFE GUVs. The first possibility is that the entire Laurdan molecule is embedded in the hydrocarbon core of PLFE liposomes with the naphthalene ring aligned parallel to the membrane surface. This configuration would give a high GP value because the hydrocarbon region of PLFE liposomes is supposed to be rigid and tight, contain-

ing no or few water channels for proton permeation (Komatsu and Chong, 1998). The observation of low average GPs (<0) in PLFE (Fig. 2) strongly argues against this possibility. Moreover, this configuration would encounter tremendous steric hindrance, because in this case Laurdan has to insert into a matrix of covalently linked biphytanoyl chains containing branched methyl groups and rigid cyclopentane rings (Fig. 1). The second possibility is that the entire Laurdan molecule is located in the polar headgroup region with both the naphthalene ring and the lauroyl chain aligned parallel to the membrane surface. This configuration would comply with the low GPs observed (Fig. 3); however, it would be energetically unfavorable for the hydrophobic lauroyl chain residing in the polar headgroup region. Hence the most plausible configuration is that the naphthalene ring resides in the PLFE polar headgroup region and the emission dipole of the Laurdan chromophore is aligned parallel to the membrane surface, while the lauroyl tail inserts into the PLFE hydrocarbon core, parallel to the

FIGURE 8 Effect of pH on the domain formation in PLFE GUVs at 12.8°C. (Left) pH 2.86. (Right) pH 7.23. Both are two-photon Laurdan fluorescence intensity images from the top of the vesicles.



PLFE phytanoyl chain. This L-shaped disposition of Laurdan explains not only the photoselection effect shown in Fig. 5, but also the low GP values presented in Fig. 3. In this disposition, the chromophore of Laurdan is in close proximity to the bound water molecules at the polar headgroups, and as a result, the degree of solvent relaxation is always extensive—hence the low GPs at all of the temperatures examined. This disposition also implies the presence of strong adhesion forces between the Laurdan's chromophore and the PLFE polar headgroups.

Laurdan's adoption of an L-shaped disposition is a consequence of the unique chemical structure and the tight packing of PLFE lipids (which accounts for the low proton permeability of the PLFE membranes; Komatsu and Chong, 1998). It is conceivable that the bulky chromophore of Laurdan (including the naphthalene ring, dimethylamino, and carbonyl groups) cannot penetrate into the PLFE hydrocarbon region because of the steric hindrance imposed by the branched methyl groups and the cyclopentane rings in the PLFE phytanoyl chains. The lauroyl chain, on the other hand, penetrates without major difficulty, as it is smaller and hydrophobic.

With the understanding that Laurdan's chromophore resides in the polar headgroup region, it can then be suggested that the small but abrupt change in GP observed at $\sim 50^\circ\text{C}$ (Fig. 3) is due to a temperature-induced conformational change of the polar headgroups in PLFE lipids. This transition temperature is in agreement with the d -spacing data of PLFE MLVs measured by small-angle x-ray diffraction (Zein et al., unpublished results), which began to exhibit an

abrupt increase when the temperature was elevated to $\sim 50^\circ\text{C}$. Unlike monopolar diester phospholipid bilayers, which have a midplane spacing between the outer and inner leaflets, bipolar tetraether PLFE liposomes have cyclopentane ring containing phytanoyl chains covalently linked from one polar end to the other, forming a monomolecular membrane. Thus the hydrocarbon region of PLFE liposomes must be rigid and is not likely to change its length with temperature to any great extent. This fact suggests that the increased d spacing at $>50^\circ\text{C}$ (Zein et al., unpublished results) most likely comes from the stretching of the polar headgroup toward the bulk solution. This conformational change would increase the exposure of Laurdan chromophore to the bulk solution, leading to increased solvent relaxation and thus a lower GP at $>50^\circ\text{C}$ (Fig. 3). Membrane lateral packing should be tighter at pH 2.86 than at pH 7.23, because of the reduction of charge repulsion between phosphate groups on PLFE lipids. The tighter membrane lateral packing would hinder the headgroup stretching discussed above. This finding explains why, at $>50^\circ\text{C}$, the GP at pH 2.86 does not drop as much as that at pH 7.23 (Fig. 3). Below 50°C , the headgroup stretching does not occur, thus giving little temperature and pH dependency for Laurdan GP (Fig. 3). An alternative explanation for the abrupt change of GP at $\sim 50^\circ\text{C}$ is that the transmembrane distribution of the PLFE polar headgroups undergoes an abrupt change at 50°C , which in turn affects the overall membrane packing, hence altering the average Laurdan solvent relaxation and the GP value. However, such a transmembrane redistribution is less likely to be the cause of the abrupt

change in GP (Fig. 3), because transmembrane redistribution should not lead to any significant alteration of membrane d spacing (Zein et al., unpublished results).

It should be noted that the abrupt change of GP in PLFE GUVs at $\sim 50^\circ\text{C}$ (Fig. 3) does not represent a gross phase transition such as the gel-to-liquid crystalline transition in bilayers composed of dipalmitoylphosphatidylcholine or other monopolar diester phospholipids. Through the main phase transition, the Laurdan excitation GP in dipalmitoylphosphatidylcholine bilayers was reported to vary from 0.6 to -0.2 (Parasassi et al., 1990). In contrast, the excitation GP in PLFE GUVs displays a much smaller change (from ~ 0 to -0.3 at pH 7.23 and from ~ 0 to -0.1 at pH 2.68) at $\sim 50^\circ\text{C}$ (Fig. 3). This small change agrees with the differential scanning calorimetry data (personal communications with E. Chang), which showed that PLFE liposomes exhibited only a broad and small-magnitude endothermic phase transition. PLFE membranes do not have the mid-plane spacing. The phytanoyl chain of PLFE contains branched methyl groups and cyclopentane rings, which are covalently linked from one polar end to the other. Such a rigid bipolar monolayer does not demonstrate the type of *gauche-to-trans* conformational transitions normally seen in bilayers composed of monopolar diester phospholipids (e.g., dipalmitoylphosphatidylcholine).

The observation of snowflake-like domains in PLFE LUVs at pH 7.23 and low temperatures (Figs. 6 and 7) is surprising. The shape of the domains seems to be regular and distinctly different from those observed for the gel state of monopolar diester phospholipids (Bagatolli and Gratton, 2000). The shape and size of the domains are stable with time, suggesting that domain formation already reaches an equilibrium on the time scale of minutes. The observation that the domains did not move significantly in the plane of the membrane at $12.8\text{--}23.2^\circ\text{C}$ (Fig. 7) suggests that PLFE liposomes are rigid and tightly packed, so little free volume is available for lateral diffusion at these temperatures. This observation is in good agreement with previous findings in cuvette studies (Kao et al., 1992; Jarrel et al., 1998). The lack of fluorescence intensity within the domains indicates that the GUVs being studied are unilamellar. For multilamellar vesicles, it would be extremely unlikely that the snowflake-like domains from one lipid layer match exactly with the domains from the others. The lack of fluorescence in the snowflake-like domains cannot be attributed to the emission dipole being aligned parallel to the phytanoyl chains, as in the case of Laurdan in the gel state of monopolar diester phospholipids (Bagatolli and Gratton, 1999, 2000). This alignment would lead to a high GP, in contradiction to the results shown in Fig. 3. Instead, what Fig. 6 shows is that Laurdan is excluded from the snowflake-like domain areas, which may be a result of an even tighter lipid packing in those domains. PLFE contains two components, GDNT ($\sim 90\%$) and GDGT ($\sim 10\%$). It is possible that GDGT separates from GDNT below certain temperatures,

forming its own lipid domains. This assertion, however, will need to be verified in the future, using vesicles composed only of GDNT.

In summary, we have demonstrated in this study that 1) stable PLFE GUVs can be readily generated by the electroformation method, 2) the Laurdan chromophore is mainly oriented parallel to the membrane surface of PLFE GUVs, 3) Laurdan's GP exhibits a small but distinct increase at $\sim 50^\circ\text{C}$, 4) Laurdan's GP distribution is wide and the GP values are small at all the temperatures and pHs examined, and 5) snowflake-like domains are formed in PLFE liposomes at low temperatures at pH 7.23. The results imply that PLFE liposomes are rigid and tightly packed and that lipid lateral separation can occur in PLFE liposomes at low temperatures. These results and implications will improve our understanding of the plasma membrane of thermoacidophilic archaeobacteria (Gliozzi and Relini, 1996), which is composed mainly of bipolar tetraether lipids. These results will also help with the development of bipolar tetraether liposomes for biotechnology applications (Ring et al., 1986; Tomioka et al., 1994; Choquet et al., 1994; Freisleben et al., 1995), all of which are based on the physical properties of the liposomes.

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