Studies of Archaebacterial Bipolar Tetraether Liposomes by Perylene Fluorescence

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ABSTRACT Membrane packing and dynamics of bipolar tetraether liposomes composed of the polar lipid fraction E (PLFE) from the thermoacidophilic archaebacterium *Sulfolobus acidocaldarius* have been studied by perylene fluorescence. At a probe-to-PLFE lipid ratio of 1:400, we have detected an unusual fluorescence intensity increase with increasing temperature, while the fluorescence lifetime changed little. As the ratio was decreased, the intensity anomaly was diminished. At 1:3200 and 1:6400, the anomaly disappeared. A remarkable perylene intensity anomaly was also observed in bilayers composed of saturated monopolar diester phosphatidylcholines at their main phase transition temperatures. These results suggest that the intensity anomaly may be due to probe aggregation caused by tight membrane packing. At the same probe-to-lipid ratio (1:400), however, 1,2-diphytanoyl-*sn*-glycero-3-phosphocholine (DPhPC) and 1,2-diphytanoyl-*sn*-glycero-3-phosphoglycerol (DPhPG) liposomes did not exhibit any intensity anomaly with increasing temperature. This suggests that DPhPC and DPhPG liposomes are more loosely packed than PLFE liposomes; thus the branched methyl groups are not the contributing factor of the tight membrane packing found in PLFE liposomes. Using a multiexcitation method, we have also determined the average (R), in-plane (R_{ip}), and out-of-plane (R_{op}) rotational rates of perylene in PLFE liposomes at various temperatures (20–65°C). R and R_{ip}, determined at two different probe-to-lipid ratios (1:400 and 1:3200), both undergo an abrupt increase when the temperature is elevated to ~48°C. These data suggest that PLFE liposomes are rigid and tightly packed at low temperatures, but they begin to possess appreciable "membrane fluidity" at temperatures close to the minimum growth temperature (~50°C) of thermoacidophilic archaebacteria.

INTRODUCTION

Thermoacidophilic archaebacterium *Sulfolobus acidocaldarius* normally grows in hot springs at 65–80°C and at pH 2–3. 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 the bipolar tetraether lipids, the polar lipid fraction E (PLFE) is the main constituent (Lo and Chang, 1990). PLFE contains a mixture of tetraether lipids with either a glycerol dialkylnonitol tetraether (GDNT) or a glycerol dialkylglycerol tetraether (GDGT) skeleton (Fig. 1). GDNT (~90% of total PLFE) contains phosphatidylmyoinositol on one end and β-glucose on the other, whereas GDGT (~10% of total PLFE) has phosphatidylmyoinositol attached to one glycerol and β-D-galactosyl-D-glucose to the other skeleton (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 temperature (De Rosa and Gambacorta, 1988).

In aqueous solutions PLFE lipids extracted from *S. acidocaldarius* form stable multilamellar and unilamellar liposomes (Lo and Chang, 1990; Elferink et al., 1992). Differential scanning calorimetric (DSC) studies showed that PLFE liposomes exhibited no or only broad and weak endothermic phase transitions (personal communications with E. Chang). Electron microscopic studies showed that in PLFE liposomes the lipids span the entire lamellar structure, forming a monomolecular thick membrane (Elferink et al., 1992). Compared to liposomes prepared from "normal" diester lipids, PLFE liposomes exhibit remarkable thermal stability in terms of the unusually low rates of proton permeation (Elferink et al., 1994; Komatsu and Chong, 1998) and carboxyfluorescein leakage (Chang, 1994; Elferink et al., 1994; Komatsu and Chong, 1998). These phenomena have been attributed to rigid and tight membrane packing and negative charges on the PLFE membrane surface (Komatsu and Chong, 1998) and have provided an explanation for why *S. acidocaldarius* can sustain a high growth temperature and live in acidic environments, while the intracellular compartment is maintained at pH 6.5.

While PLFE lipids provide archaebacteria with a rigid and tight barrier between the intracellular and extracellular environments, they must also provide archaebacterial membranes with some "fluidity" to exhibit functionality (In't Veld et al., 1992; Elferink et al., 1993). To understand archaebacterial "membrane fluidity," researchers have investigated the lateral and rotational diffusions of membrane components in bipolar tetraether liposomes. The lateral mobility of a pyrene-labeled phosphatidylcholine in *S. acidocaldarius* PLFE liposomes was found to be highly restricted and only became appreciable at temperatures higher than 48°C (Kao et al., 1992). This conclusion has recently been confirmed and elaborated by a ^{31}P-NMR study on tetraether

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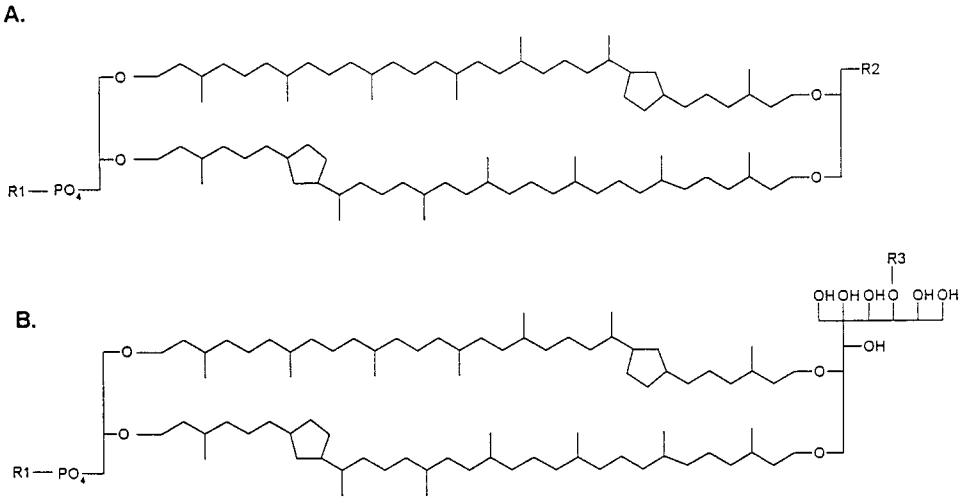

FIGURE 1 Structures of PLFE lipids. (*A*) Glycerol dialkylglycerol tetraether (GDGT). (*B*) Glycerol dialkylnonitol tetraether (GDNT). R1 = inositol; R2 = β-D-glucopyranose; R3 = β-D-galactosyl-β-D-glucopyranose. The number of cyclopentane rings can vary from 0 to 4 in each phytanyl chain (De Rosa and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

liposomes from *Thermoplasma acidophilum*, a thermoacidophilic archaebacterium grown at pH 2 and 55–59°C (Jarrel et al., 1998). The rotational correlation times of spin labels at different positions of the hydrocarbon chain of tetraether lipids from *S. solfataricus* (a thermoacidophilic archaebacterium) were found to vary with water content and the depth of the probe from the membrane surface (Bruno et al., 1985). The spin label study also showed that, even at high temperatures (~85°C), the nonitol headgroup was relatively immobile and the rotation of the probe was still anisotropic (Bruno et al., 1985). Despite these efforts, more quantitative assessments of archaebacterial "membrane fluidity" are in demand. For example, electron spin resonance measures molecular motions on the time scale of 10^{-6}–10^{-7} s. To detect faster rotational motions, fluorescence spectroscopy is more appropriate. There have been other fluorescence studies of archaebacterial lipid membranes; however, they were confined to steady-state measurements and dynamic information was not reported (Lelkes et al., 1983).

In this work we have used nanosecond fluorescence probe techniques to characterize the dynamic structures of *S. acidocaldarius* PLFE liposomes. Perylene, a commonly used membrane probe, was employed to explore PLFE membrane packing and dynamics. Using the multiexcitation method (Chong et al., 1985), we have determined the average, in-plane, and out-of-plane rotational rates of perylene in PLFE liposomes at various temperatures (20–65°C). Our data indicate that the rotational motions of perylene in the hydrocarbon regions of PLFE liposomes increase abruptly at ~48°C, a temperature close to the minimum growth temperature (~50°C) of thermoacidophilic archaebacteria (Gliozzi and Relini, 1996). Moreover, we have used the perylene fluorescence intensity anomaly to conduct comparative studies of membrane packing between PLFE liposomes and various nonarchaebacterial liposomes and suggested that 1,2-diphytanoyl-*sn*-glycero-3-phosphocholine (DPhPC) and 1,2-diphytanoyl-*sn*-glycero-3-phosphoglycerol (DPhPG) liposomes are loosely packed compared to PLFE liposomes.

MATERIALS AND METHODS

Materials

S. acidocaldarius cells (strain DSM639; American Type Culture Collection, Rockville, MD) were grown aerobically and heterotrophically at 69–70°C, pH 2.5–3.0. The growth was monitored by absorbance at 420 and 540 nm. 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 with 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, Clifton, 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.

1,2-Dimyristoyl-*sn*-glycero-3-phosphocholine (DMPC), 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC), 1,2-distearoyl-*sn*-glycero-3-phosphocholine (DSPC), 1,2-diphytanoyl-*sn*-glycero-3-phosphocholine (DPhPC), and 1,2-diphytanoyl-*sn*-glycero-3-phosphoglycerol (DPhPG) were purchased from Avanti Polar Lipids (Alabaster, AL). Perylene was obtained from Molecular Probes (Eugene, OR). The concentration of perylene stock solution (in chloroform) was determined using an extinction coefficient at 410 nm equal to 35,000 M^{-1} cm^{-1} (in ethanol) (Jacobson and Wobschall, 1974). The concentration of phospholipids was determined according to the method of Bartlett (1959).

Liposome preparation

PLFE multilamellar vesicles labeled with perylene were prepared as follows. In the first step, PLFE lipids (1.56 mg dry powder) were dissolved in chloroform:methanol (1:1, v/v), and an appropriate amount of perylene in chloroform was added and mixed well with the PLFE solution. The mixture was evaporated to dryness under nitrogen and then under high vacuum for ≥12 h. In the second step, the mixture was suspended in chloroform:

methanol:water (65:25:10, v/v) (Lo and Chang, 1990) and evaporated to dryness under nitrogen and then under high vacuum overnight. To the dried perylene/PLFE mixture, 3.5 ml of phosphate buffer (50 mM, pH 7.2) was added. The mixture was vigorously vortexed at 65°C for 12 min and then incubated for >4 h at 65°C before fluorescence measurements. For the blank the same procedure was used, except that perylene was not included. Perylene-labeled nonarchaebacterial liposomes were prepared either by the two-step method mentioned above or by the two-step method without the suspension in chloroform:methanol:water (65:25:10, v/v).

Measurements of fluorescence intensity, anisotropy, and lifetime

Fluorescence intensity measurements were made with an SLM 8000C fluorometer (Urbana, IL), using various excitation wavelengths (1-nm band-pass). Emission was collected through a monochromator with an 8-nm band-pass. Steady-state anisotropy measurements were made on an ISS K-2 fluorometer (Champaign, IL), using an L-format optical arrangement. Fluorescence lifetimes were determined on an ISS K-2 multifrequency phase modulation fluorometer. For the lifetime measurements, the excitation light was modulated with a Pockels cell, the excitation polarizer was set at 35° with respect to the vertical plane, and no emission polarizer was used. Phase and modulation values were determined relative to a *p*-bis[2-(5-phenyloxazolyl)]benzene (POPOP) (in ethanol) reference solution, which has a lifetime of 1.35 ns (Lakowicz et al., 1981). The data of emission were analyzed with the software provided by ISS Inc., based on the scheme described by Gratton et al. (1984). In brief, the data were fit by a multiexponential decay law, $F(t) = \Sigma\alpha_i \exp(-t/\tau_i)$, where α_i and τ_i are the preexponential factor and lifetime for the ith component, respectively. The goodness of the fit was determined by the reduced χ^2. The average lifetime $\langle\tau\rangle$ was calculated from the equation $\langle\tau\rangle = \Sigma f_i\tau_i$, where f_i is the fractional intensity of the ith component.

For all of the fluorescence measurements, the temperature of the samples was controlled by a circulating bath, the light source was a xenon arc lamp, and the samples were measured while stirring. For intensity and anisotropy measurements, the blank readings from membranes without probes were subtracted from the sample readings. Perylene emission for anisotropy and lifetime measurements was collected through a Schott KV450 cutoff filter.

Determinations of R, R_{ip}, and R_{op} of perylene by the multiexcitation method

Perylene is a flat aromatic molecule with a rigid, disk-like structure. Assume that the z axis is perpendicular to the ring system and that the x axis is along the emission dipole moment, whereas the absorption dipole moment lies also in the plane of the ring system at an angle α with respect to the x axis (Chong et al., 1985). Then perylene has two principal modes of rotation: about the z axis (in plane) and about an axis located in the plane of the ring system (out of plane). Such an anisotropic perylene rotation has previously been observed in micelles (Shinitzky et al., 1971), lipid bilayers (Cogan et al., 1973; Chong et al., 1985; Lakowicz et al., 1985), and isotropic media such as propylene glycol (Mantulin and Weber, 1977; Barkley et al., 1981).

In the present study, the rate of in-plane rotation (R_{ip}) and the rate of out-of-plane rotation (R_{op}) in PLFE liposomes were determined as a function of temperature by a multiexcitation method (Chong et al., 1985). This method considers not only the rotational hindrance but also the nonisotropic nature of perylene rotation and takes advantage of the fact that the angle between emission and absorption, α, can be varied from 22° to 90° by appropriately choosing the excitation wavelength in the 270–410-nm range. When the fundamental anisotropy, r_o, is varied by changing the excitation wavelength, a linear plot of the steady-state anisotropy, r, versus $(r_o - r)/\langle\tau\rangle$ can be generated according to Eq. 1 (Chong et al., 1985):

$$r = r_i + (r_o - r)/6R\langle\tau\rangle \quad (1)$$

where

$$r_i = \frac{0.1[(1+\xi)(1-S^2) + (1-\xi)(S^2 + 6S^2R\langle\tau\rangle - 1)]}{(1+\xi)(1-S^2) + (1-\xi)6R\langle\tau\rangle} \quad (2)$$

$$\xi = \text{rotational anisotropy} = (R_{ip} - R_{op})/(R_{ip} + 2R_{op}) \quad (3)$$

$$S = \text{order parameter} = (10r_\infty)^{1/2} = (3\cos^2\theta - 1)/2 \quad (4)$$

The limiting fluorescence anisotropy, r_∞, is the anisotropy at times much longer than the rotational correlation times of the fluorophore, and θ is the angle between the molecular z axis and the membrane normal. The slope of the plot equals $1/(6R)$, where R is the average rotational rate and the intercept equals r_i. Thus R can be obtained and the allowed region in the (ξ,S) plane can be determined. The (ξ,S) plane was established by using Eq. 2 with the calculated R and r_i values, and with the measured $\langle\tau\rangle$ values for $-1/2 \le S \le 0$ (i.e., $54.76° \le \theta \le 90°$) (Chong et al., 1985). Here the x-y plane of perylene presumably aligns parallel to the membrane normal; therefore, the most probable θ is 90°, and a negative order parameter S is expected (Zannoni et al., 1983). Finally, R_{ip} and R_{op} can be calculated by using Eqs. 5 and 6, and their errors are estimated from the lower and upper limits of ξ and R (Chong et al., 1985):

$$R_{ip} = (1 + 2\xi)R/(1 + \xi) \quad (5)$$

$$R_{op} = (1 - \xi)R/(1 + \xi) \quad (6)$$

RESULTS AND DISCUSSION

Intensity anomaly

Fig. 2 illustrates the temperature dependence of the excitation and emission spectra of perylene in PLFE liposomes (perylene/PLFE ~1:400). While the spectrum shape and the band position are temperature-invariant, the intensity increases considerably with increasing temperature (~33% from 20°C to 65°C; Fig. 3 *A, filled circles*). This intensity

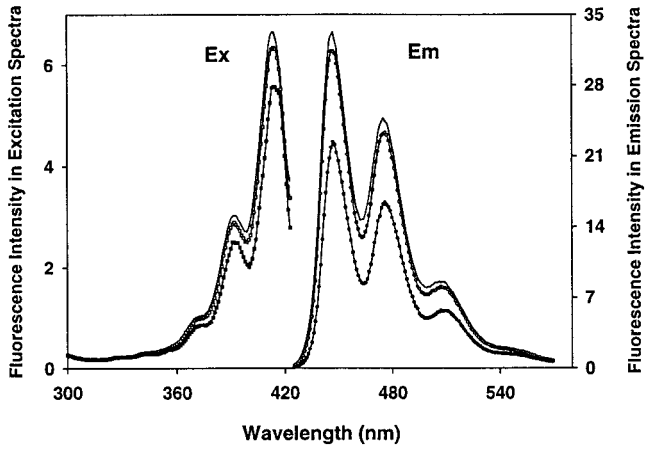

FIGURE 2 Illustration of the temperature dependence of excitation spectra (λ_{em} = 450 nm) and corrected emission spectra (λ_{ex} = 410 nm) of perylene in PLFE liposomes. Perylene/PLFE = 1:400. Excitation: 14.9°C (■), 52.3°C (□), and 70.2°C (——). Emission: 16.7°C (●), 49.6°C (○), and 70.1°C (——).

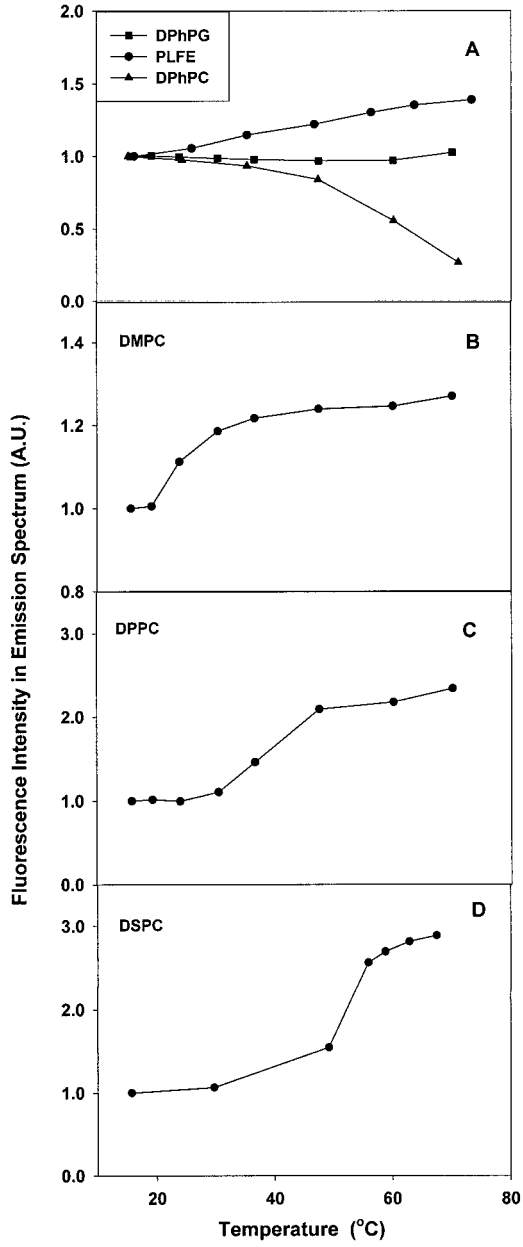

FIGURE 3 The temperature dependence of the increase of the normalized perylene fluorescence intensity in PLFE (*A*, ●), DPhPG (*A*, ■), DPhPC (*A*, ▲), DMPC (*B*), DPPC (*C*), and DSPC (*D*) liposomes. Perylene/PLFE = 1:400. In each sample, the intensity at the lowest temperature examined was normalized to unity.

increase is abnormal because the fluorescence intensity of membrane probes normally decreases with increasing temperature as a result of enhanced quenching processes. The anomalous behavior of perylene fluorescence intensity was observed not only in PLFE liposomes (Figs. 3 *A*), but also in vesicles composed of nonarchaebacterial lipids such as DMPC, DPPC, and DSPC (Papahadjopoulos et al., 1973; Figs. 3, *B–D*), as well as in muscle microsomal membranes (Rubsamen et al., 1976).

To explain the intensity anomaly, it was previously proposed (Papahadjopoulos et al., 1973) that perylene resides at two different sites: the hydrocarbon interior (high fluorescence quantum yield) and the membrane-water interfacial region (low quantum yield). At low temperatures, some perylene molecules are excluded from the hydrocarbon interior of the membrane because of tight lipid packing, but they are inserted back into the hydrocarbon core at high temperatures. Based on this explanation, it is predicted that the fluorescence lifetime of perylene in PLFE liposomes increases with increasing temperature, analogous to the intensity change. However, the average fluorescence lifetime (measured with λ_{ex} = 410 nm) of perylene in PLFE liposomes increases by only ~5% from 20°C to 65°C (Fig. 5 *A*), which is not comparable to the intensity change (~33%) over the same temperature range examined (Fig. 3 *A, filled circles*). Thus the previous interpretation of the perylene intensity anomaly must be modified.

It is likely that some perylene molecules self-aggregate in the membrane (Rubsamen et al., 1976), forming nonfluorescent species as a result of self energy transfer, while others embedded in the membrane in the monomeric form readily fluoresce. As temperature is elevated, aggregated perylene increases its dissociation, leading to an increase in fluorescence intensity. The proposal that aggregated perylene has a fluorescence lifetime close to 0 ns explains why the average lifetime of perylene fluorescence in PLFE liposomes changes relatively little with temperature (Fig. 5 *A*). Furthermore, note that, after passing through a Sephadex G-50 column, perylene/PLFE liposomes still exhibit the intensity anomaly (data not shown). This suggests that the aggregated species reside in the membrane rather than in the aqueous phase and that the anomaly is not caused by the

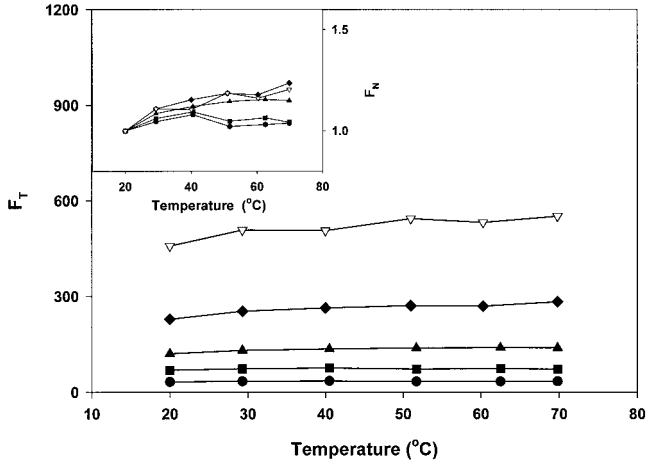

FIGURE 4 Effect of the probe-to-lipid ratio (▽, 1:400; ◆, 1:800; ▲, 1:1600; ■, 1:3200; ●, 1:6400) on the temperature dependence of the unnormalized total intensity of perylene fluorescence (F_T) in PLFE liposomes. *Insets:* Plots of the normalized intensity (F_N) versus temperature at different probe-to-lipid ratios.

TABLE 1 Illustrations of fitted decay parameters of perylene fluorescence in PLFE multilamellar vesicles at various temperatures

Temp. (°C)	λ_{ex} (nm)	τ_1 (ns)	τ_2 (ns)	f_1	χ^2	$\langle\tau\rangle$ (ns)
Perylene/PLFE ~1:400						
34.3	330	7.78 ± 0.08	1.82 ± 0.18	0.80 ± 0.01	3.6	6.57 ± 0.07
	340	9.25 ± 0.13	3.59 ± 0.08	0.63 ± 0.01	3.8	7.14 ± 0.09
	350	7.13 ± 0.06	2.94 ± 0.11	0.79 ± 0.01	2.5	6.26 ± 0.05
	360	8.32 ± 0.14	3.91 ± 0.06	0.52 ± 0.01	3.5	6.18 ± 0.08
	370	6.26 ± 0.02	2.73 ± 0.11	0.86 ± 0.01	0.9	5.78 ± 0.02
	380	5.74 ± 0.04		1.00	2.8	5.74 ± 0.04
	390	5.69 ± 0.04		1.00	3.6	5.69 ± 0.04
	400	5.31 ± 0.04		1.00	3.3	5.31 ± 0.04
	410	5.05 ± 0.05		1.00	3.6	5.05 ± 0.05
50.6	330	8.04 ± 0.07	3.96 ± 0.08	0.70 ± 0.01	2.2	6.80 ± 0.05
	340	6.57 ± 0.03	0.01 ± 0.05	0.89 ± 0.01	1.5	5.83 ± 0.03
	350	6.37 ± 0.04	0.50 ± 0.14	0.91 ± 0.01	3.2	5.82 ± 0.04
	360	5.94 ± 0.04		1.00	3.9	5.94 ± 0.04
	370	6.04 ± 0.05		1.00	3.7	6.04 ± 0.05
	380	5.87 ± 0.05		1.00	5.5	5.87 ± 0.05
	390	5.58 ± 0.04		1.00	3.5	5.58 ± 0.04
	400	5.70 ± 0.04		1.00	3.4	5.70 ± 0.04
	410	5.47 ± 0.04		1.00	3.9	5.47 ± 0.04
64.7	330	8.33 ± 0.07	3.06 ± 0.09	0.74 ± 0.01	2.5	6.96 ± 0.06
	340	7.55 ± 0.06	2.85 ± 0.12	0.82 ± 0.01	2.3	6.72 ± 0.05
	350	6.19 ± 0.05		1.00	4.2	6.19 ± 0.05
	360	5.94 ± 0.04		1.00	2.7	5.94 ± 0.04
	370	5.93 ± 0.04		1.00	3.9	5.93 ± 0.04
	380	5.95 ± 0.04		1.00	2.4	5.95 ± 0.04
	390	5.74 ± 0.04		1.00	2.8	5.74 ± 0.04
	400	5.69 ± 0.05		1.00	4.7	5.69 ± 0.05
	410	5.47 ± 0.05		1.00	4.6	5.47 ± 0.05
Perylene/PLFE ~1:3200						
34.3	380	7.54 ± 0.16	1.80 ± 0.18	0.69 ± 0.01	5.8	5.76 ± 0.12
	390	6.10 ± 0.04	0.17 ± 0.03	0.80 ± 0.00	0.8	4.91 ± 0.04
	400	7.13 ± 0.05	2.06 ± 0.06	0.71 ± 0.00	1.5	5.40 ± 0.06
	410	5.85 ± 0.03	1.17 ± 0.17	0.76 ± 0.00	1.7	4.73 ± 0.05
50.2	380	6.69 ± 0.06	1.13 ± 0.37	0.79 ± 0.01	4.7	5.52 ± 0.09
	390	6.68 ± 0.05	2.07 ± 0.15	0.82 ± 0.01	2.7	5.85 ± 0.05
	400	5.93 ± 0.04	0.42 ± 0.11	0.87 ± 0.01	3.6	5.21 ± 0.04
	410	5.72 ± 0.05	1.32 ± 0.24	0.78 ± 0.01	4.8	4.75 ± 0.07

increased partitioning of free perylene into the membrane at higher temperatures. A decrease in the probe-to-lipid ratio reduces the total fluorescence intensity and the extent of the intensity anomaly in PLFE liposomes (Fig. 4). At ratios of 1:3200 and 1:6400 the intensity anomaly disappears (Fig. 4). These results support the assertion that the intensity anomaly is caused mainly by probe aggregation.

The above explanation is supported by the observation that the intensity anomaly varies with the physical state of lipid membranes. As shown in Fig. 3, *B–D*, the normalized fluorescence intensity of perylene in DMPC, DPPC, and DSPC multilamellar vesicles (probe-to-lipid ratio ~1:400) increases sharply at ~22, 38, and 53°C, respectively. These temperatures come close to the main phase transition temperatures of the corresponding matrix lipids (23°C for DMPC, 41.5°C for DPPC, and 54.5°C for DSPC; Marsh, 1990). This makes sense because when membrane packing is tight in the gel state of phospholipids (at low temperatures), most perylene molecules would be aligned parallel to the molecular axis of lipid acyl chains (Zannoni et al., 1983). This parallel orientation would favor the formation of staggered aggregates of perylene (e.g., Bevers et al., 1998). In contrast, in the liquid-crystalline state (at high temperatures), membrane packing becomes loose, and, as a result, perylene is more randomly oriented. The less ordered orientation plus the thermal-induced dissociation of perylene aggregates at high temperatures would increase the number of nonaggregated perylenes. This explains why there is a sharp increase in perylene fluorescence intensity during the main phase transition of DMPC, DPPC, and DSPC bilayers (Figs. 3, *B–D*). By the same token, the lack of a sharp intensity increase with increasing temperature in PLFE liposomes (Fig. 3 *D*) can be taken to indicate that there is no distinct phase transition in the temperature range examined, a scenario in agreement with previous DSC studies (personal communications with E. Chang).

In contrast to the cases of PLFE, DMPC, DPPC, and DSPC, the perylene fluorescence intensity anomaly does

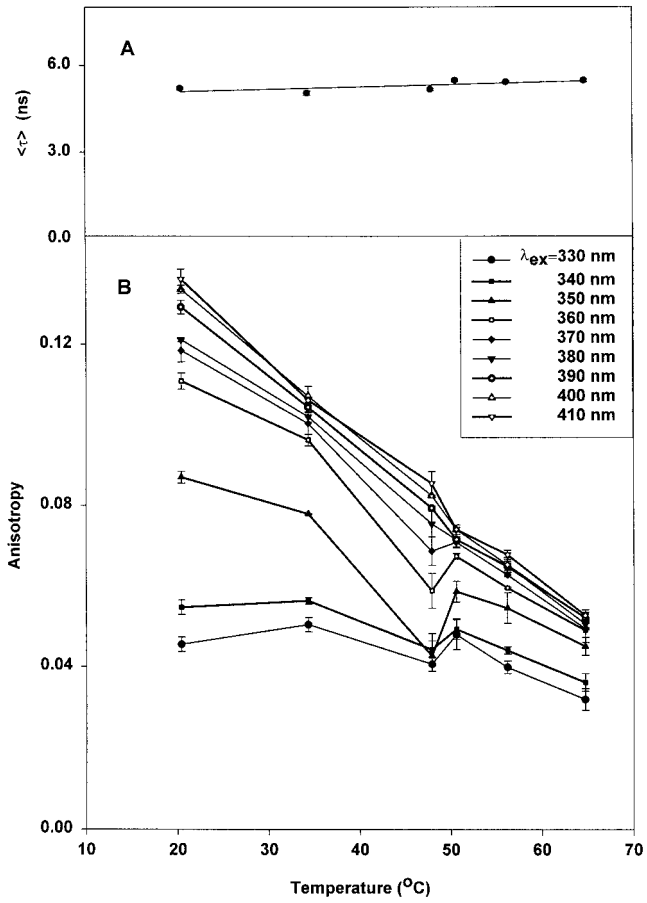

FIGURE 5 The temperature dependence of (*A*) the average lifetime $\langle\tau\rangle$ with λ_{ex} = 410 nm and (*B*) the steady-state anisotropy of perylene fluorescence in PLFE liposomes. Perylene/PLFE = 1:400.

not appear in DPhPC and DPhPG liposomes (Fig. 3 *A*, probe-to-lipid ratio ~1:400), both of which contain branched methyl groups in the hydrocarbon region. In DPhPC, the fluorescence intensity of perylene actually decreases monotonically with increasing temperature (Fig. 3 *A, filled triangles*). DPhPC does not have a phase transition from −120°C to 80°C (Silvius, 1992). Taken together, our result suggests that, at low temperature, membrane packing of DPhPC liposomes is already loose and few perylene molecules are aggregated. An increase in temperature does not cause any appreciable change in the population of nonaggregated perylene; instead, it mainly increases quenching processes, resulting in an intensity decrease. The conclusion, that at low temperature membrane packing in DPhPC liposomes is loose compared to that in PLFE liposomes, is supported by molecular modeling calculations (Gabriel and Chong, unpublished results).

Lifetime and anisotropy data

The temperature dependence of perylene fluorescence lifetimes in PLFE liposomes was measured at various excitation wavelengths (330–410 nm for perylene/PLFE ~1:400 and 380–410 nm for perylene/PLFE ~1:3200). At a probe-to-PLFE lipid ratio of 1:400, the data obtained from short excitation wavelengths are best fit with a two-exponential decay law, whereas the data obtained from long excitation wavelengths are best described by a single-exponential decay, as illustrated in Table 1. But as the temperature increases, the $\lambda_{ex,}$ that divides the single-exponential fits and the double-exponential fits moves toward the shorter wavelengths (Table 1). At a probe-to-PLFE lipid ratio of 1:3200, however, the data obtained from long excitation wavelengths (380–410 nm) are best fit with a two-exponential decay law (Table 1). Because of weak signals, the lifetime measurements at short excitation wavelengths (<380 nm) were not performed for samples with a probe-to-lipid ratio of 1:3200. Although the physical origin for the differences in fluorescence decay parameters at different probe-to-lipid ratios is not understood, the average lifetime, $\langle\tau\rangle$, of perylene fluorescence in PLFE liposomes does not seem to vary much with the probe-to-lipid ratio (Table 1). At any given probe-to-lipid ratio and λ_{ex}, $\langle\tau\rangle$ varies little with temperature (as illustrated in Figs. 5 *A* and 6 *A*). The lack of a sharp change in $\langle\tau\rangle$ with temperature indicates again the absence of a gross phase transition in PLFE liposomes in the temperature range examined.

When excited at 350 ± 20 nm, the steady-state anisotropy of perylene fluorescence in PLFE liposomes (probe/lipid ~1:400) shows an abrupt change at ~48°C (Fig. 5 *B*).

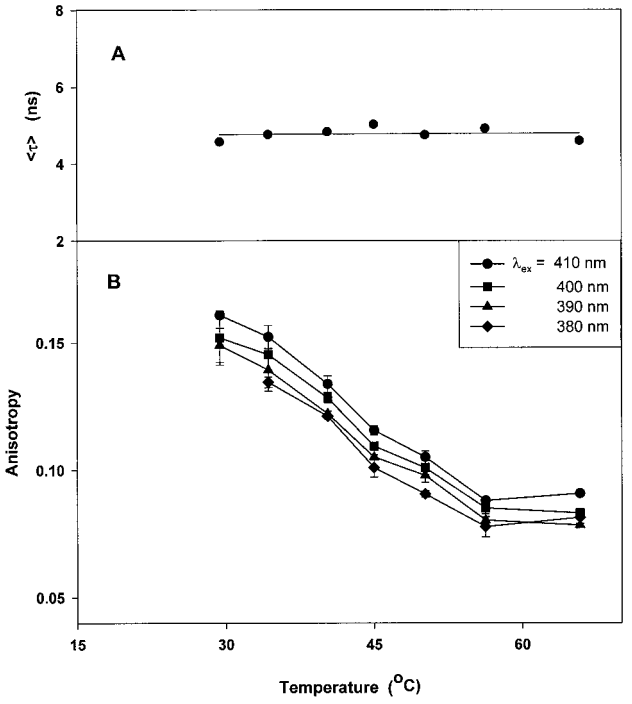

FIGURE 6 The temperature dependence of (*A*) the average lifetime $\langle\tau\rangle$ with λ_{ex} = 410 nm and (*B*) the steady-state anisotropy of perylene fluorescence in PLFE liposomes. Perylene/PLFE = 1:3200.

Because the anisotropy mainly reports the free volume in the vicinity of the probe, the abrupt change might reflect a local structural change in the PLFE hydrocarbon core (discussed later). According to Weber (1971) and Shinitzky et al. (1971), when $\alpha = 90°$ (or $r_o = -0.25$), the observed depolarization is due only to the rotation in the plane of perylene, whereas at $\alpha = 45°$ (or $r_o = 0.1$) depolarization is due only to the out-of-plane rotation. It can be estimated from the data of Chong et al. (1985) that $r_o = 0.1$ at $\lambda_{ex} = 315$ nm and $r_o = -0.25$ at $\lambda_{ex} = 250$ nm. Thus the abrupt anisotropy change at ~48°C observed at $\lambda_{ex} = 350 \pm 20$ nm (Fig. 5 *B*) is not due only to the in-plane or the out-of-plane rotation, but a combination of both.

Determinations of R, R_{ip}, and R_{op}

Perylene/PLFE = 1:400

Using the r_o values previously determined (Chong et al., 1985) and the steady-state anisotropy (r) (Fig. 5 *B*), as well as the average lifetime ($\langle\tau\rangle$) (Table 1) determined in this study, we have constructed a plot of r versus $(r_o - r)/\langle\tau\rangle$ for each temperature employed (Fig. 7). In all cases, the data are fitted to a straight line, and the slope of the plot yields the average rotational rate, R, according to Eq. 1. As shown in Fig. 8 *A* and Table 2, R for perylene in PLFE liposomes (perylene/PLFE ~1:400) steadily increases with increasing temperature up to ~48°C, where it undergoes an abrupt increase.

Using the values of R, the intercepts of the plots in Fig. 7, and the multiexcitation method described earlier, we have calculated the R_{ip} and R_{op} values of perylene in PLFE liposomes (probe/lipid ~1:400) as a function of temperature. It is clear from Fig. 8 *B* that R_{ip} undergoes an abrupt increase at ~48°C, similar to the case of R (Fig. 8 *A*). Because R_{op} is typically associated with relatively large errors (Chong et al., 1985; Lakowicz et al., 1985), it is not certain whether there is an abrupt change in R_{op} at ~48°C, but it can still be concluded from Fig. 8 *C* that R_{op} increases slightly with increasing temperature. The Arrhenius plot of R_{ip} (not shown) also shows a break point at ~48°C. The

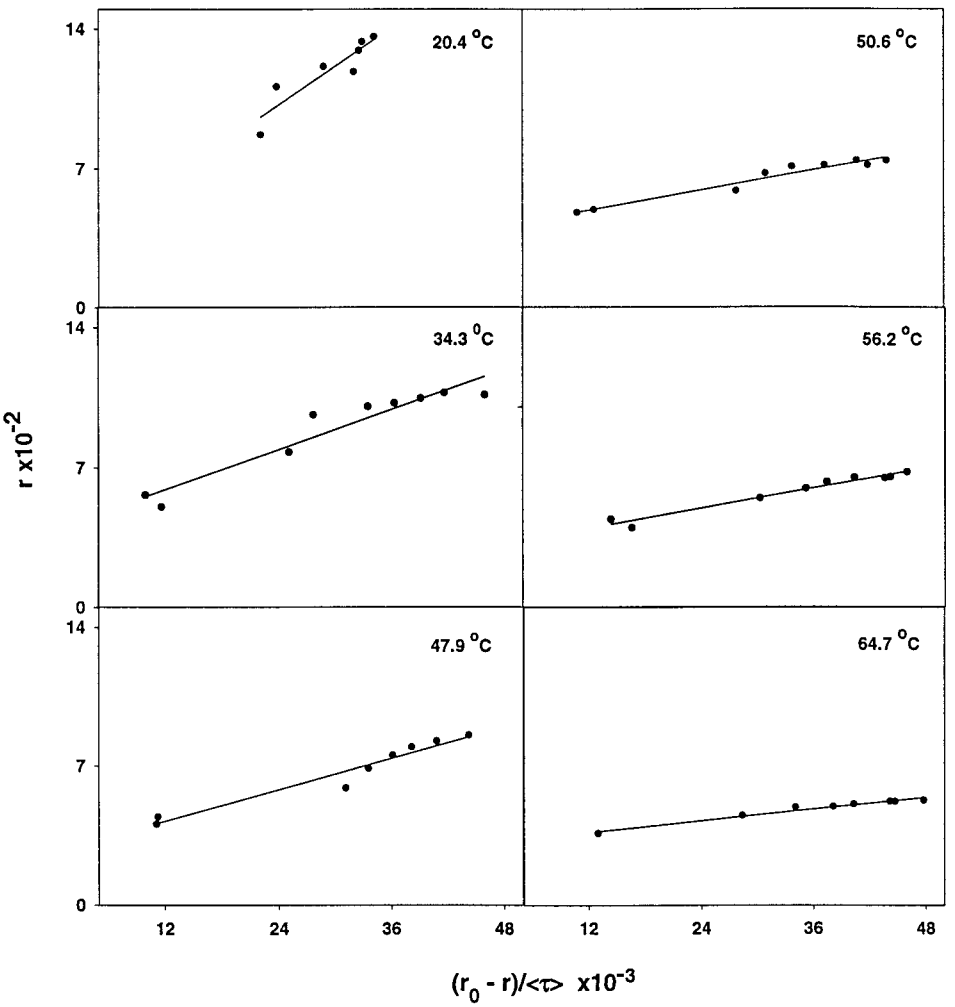

FIGURE 7 The steady-state fluorescence anisotropy, r, as a function of $(r_o - r)/\langle\tau\rangle$ for perylene in PLFE liposomes at various temperatures. Perylene/PLFE = 1:400.

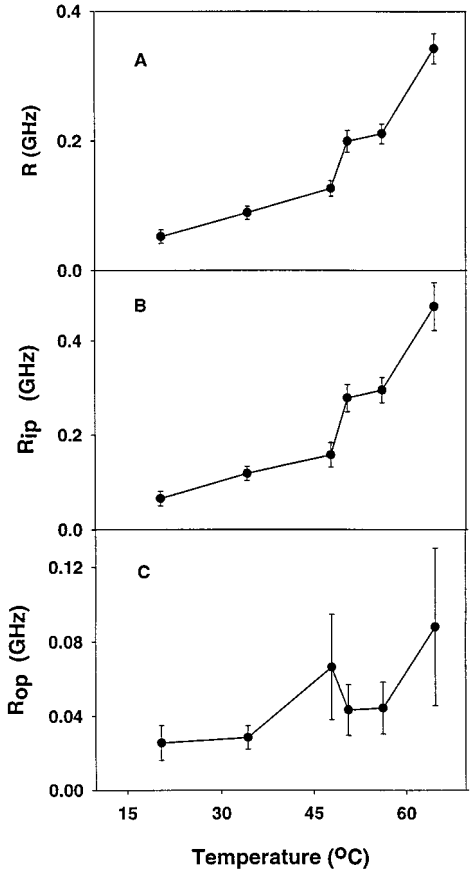

FIGURE 8 Effect of temperature on (*A*) the average rotational rate, *R*; (*B*) the rate of in-plane rotation, R_{ip}; and (*C*) the rate of out-of-plane rotation, R_{op}, of perylene in PLFE liposomes. Perylene/PLFE = 1:400.

activation energy of R_{ip} is estimated to be 35.4 ± 11.3 kJ/mol above 48°C and 25.0 ± 4.2 kJ/mol below 48°C. As shown in Table 2 and Fig. 8, at any given temperature, R_{ip} is much greater than R_{op}, in agreement with previous findings in nonarchaebacterial liposomes (Lakowicz and Knutson, 1980; Chong et al., 1985) and in paraffin (Zinsli, 1977).

TABLE 2 Rates of different rotational motions of perylene in PLFE liposomes at various temperatures

Perylene/ PLFE	Temp. (°C)	R ± SD (GHz)	R_{ip} ± SD (GHz)	R_{op} ± SD (GHz)
1:400	20.4	0.052 ± 0.010	0.066 ± 0.015	0.026 ± 0.009
	34.3	0.089 ± 0.010	0.119 ± 0.015	0.029 ± 0.006
	47.9	0.126 ± 0.012	0.158 ± 0.026	0.066 ± 0.028
	50.6	0.199 ± 0.017	0.278 ± 0.029	0.043 ± 0.014
	56.2	0.211 ± 0.015	0.295 ± 0.027	0.044 ± 0.014
	64.7	0.343 ± 0.024	0.473 ± 0.051	0.088 ± 0.042
1:3200	29.4	0.064 ± 0.018	0.091 ± 0.026	0.049 ± 0.044
	34.3	0.077 ± 0.037	0.113 ± 0.054	0.006 ± 0.003
	40.3	0.076 ± 0.027	0.106 ± 0.039	0.018 ± 0.008
	45.0	0.085 ± 0.024	0.112 ± 0.034	0.033 ± 0.014
	50.2	0.197 ± 0.006	0.289 ± 0.010	0.013 ± 0.001
	65.8	0.216 ± 0.041	0.311 ± 0.061	0.026 ± 0.008

This trend is reasonable, as the out-of-plane rotation requires a larger volume change than the in-plane rotation.

Perylene/PLFE = 1:3200

We have repeated the above rotational rate measurements, using samples with a perylene-to-PLFE ratio of ~1:3200. At this low ratio, the perylene intensity anomaly was not found (Fig. 4), so the changes in rotational parameters cannot be attributed to probe aggregation (discussed earlier). The drawback of using this low ratio is that the fluorescence signal becomes too weak at low excitation wavelengths (330–370 nm) (Fig. 1). As a result, we have restricted the use of the multiexcitation method to a shorter excitation wavelength region, namely, 380–410 nm. The results from the samples with perylene/PLFE ~1:3200 are shown in Fig. 9 and Table 2. At a perylene/PLFE ratio of 1:3200, R and R_{ip} exhibit an abrupt change at 45–50°C, a

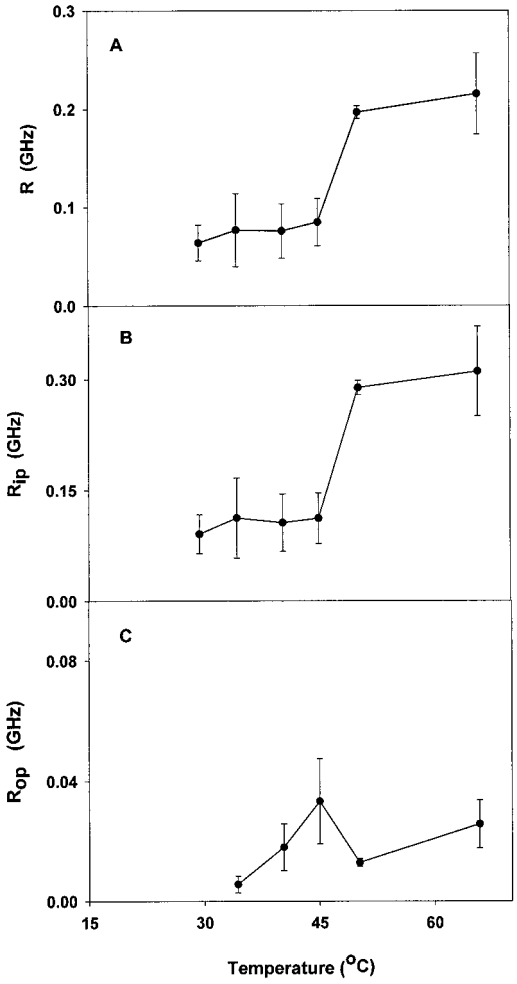

FIGURE 9 Effect of temperature on (*A*) the average rotational rate, *R* ; (*B*) the rate of in-plane rotation, R_{ip}; and (*C*) the rate of out-of-plane rotation, R_{op}, of perylene in PLFE liposomes. Perylene/PLFE = 1:3200.

result consistent with that obtained from the high probe-to-lipid ratio (1:400).

Structural and functional implications

Regardless of the probe-to-lipid ratio, both R, and R_{ip} show an abrupt change at ~48°C. This result is consistent with the previous study of the lateral mobility of pyrene-labeled phosphatidylcholine in PLFE liposomes (Kao et al., 1992), which showed little probe lateral mobility until 48°C. This temperature, however, does not correspond to a gross phase transition (such as the gel-to-liquid crystalline transition of DMPC, DPPC, or DSPC), according to the DSC data (personal communications with E. Chang). Unlike bilayers composed of monopolar diester phospholipids (e.g., DMPC, DPPC, and DSPC), PLFE membranes do not have the midplane 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 bipolar monolayers do not have the type of *gauche*-to-*trans* conformational transition normally seen in bilayers composed of monopolar lipids.

The abrupt change in perylene rotational parameters at ~48°C may represent a change in PLFE membrane packing at the areas where the probe perylene resides. Perylene is a hydrophobic compound without any hydrophilic side chains. Therefore, it is unlikely that perylene would reside in the PLFE polar headgroup region. Perylene is a flat and disk-like molecule with a size (~51 Å^2) comparable to the cross-sectional area (56–58 Å^2) of the hydrocarbon region in a typical bipolar tetraether lipid (Gulik et al., 1988). Thus it is also unlikely that perylene would reside in the hydrocarbon region of PLFE liposomes with its *x-y* plane aligned parallel to the membrane surface. The most plausible disposition is that perylene is embedded in the PLFE hydrocarbon region with the *x-y* plane parallel to the membrane normal. With this probe disposition in mind, it can be suggested that the cyclopentane rings and the branched methyl groups of PLFE lipids provide a steric hindrance for the in-plane rotation of perylene at low temperatures. For some reason, such steric hindrance is alleviated at temperatures above ~48°C, consequently causing an abrupt increase in the in-plane rotation of perylene (Figs. 8 and 9). Although at present the reason behind the abrupt change in R_{ip} at ~48°C is not clearly understood, we do have supporting evidence that relates the abrupt change in R_{ip} to the static structural change in PLFE liposomes. Our recent small-angle x-ray diffraction data on PLFE multilamellar vesicles showed a small but distinct change in d-spacing at ~50°C (Zein, Winter, Khan, and Chong, unpublished results), which is close to the temperature for the abrupt change in R_{ip} (Figs. 8 and 9).

Perhaps, through the dynamic structural change demonstrated in this study, the plasma membrane of *S. acidocaldarius*, where PLFE is the major component, begins to gain sufficient "fluidity" for functionality (In't Veld et al., 1992; Elferink et al., 1993) at ~48°C. Interestingly, this temperature is close to the minimum growth temperature of thermoacidophilic archaebacteria (~50°C) (Gliozzi and Relini, 1996). This point may be of fundamental importance in understanding the structure-function relationship of archaebacterial membranes and may help the development of bipolar tetraether liposomes for applications in biotechnology. Bipolar tetraether liposomes can be used for sterilization (Choquet et al., 1994), immunoassays (Tomioka et al., 1994), drug delivery (Ring et al., 1986; Sprott, 1992; Elferink et al., 1994: Freisleben et al., 1995), and the reconstitution study of channel-forming proteins or peptides.

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