Triton X-100 Partitioning into Sphingomyelin Bilayers at Subsolubilizing Detergent Concentrations: Effect of Lipid Phase and a Comparison with Dipalmitoylphosphatidylcholine

Cristina Arnulphi, Jesús Sot, Marcos García-Pacios, José-Luis R. Arrondo, Alicia Alonso, and Félix M. Goñi
Unidad de Biofísica (Centro Mixto CSIC-UPV/EHU) and Departamento de Bioquímica, Universidad del País Vasco, 48080 Bilbao, Spain

ABSTRACT We examined the partitioning of the nonionic detergent Triton X-100 at subsolubilizing concentrations into bilayers of either egg sphingomyelin (SM), palmitoyl SM, or dipalmitoylphosphatidylcholine. SM is known to require less detergent than phosphatidylcholine to achieve the same extent of solubilization, and for all three phospholipids solubilization is temperature dependent. In addition, the three lipids exhibit a gel-fluid phase transition in the 38–41°C temperature range. Experiments have been performed at Triton X-100 concentrations well below the critical micellar concentration, so that only detergent monomers have to be considered. Lipid/detergent mol ratios were never <10:1, thus ensuring that the solubilization stage was never reached. Isothermal titration calorimetry, DSC, and infrared, fluorescence, and ^{31}P-NMR spectroscopies were applied in the 5–55°C temperature range. The results show that, irrespective of the chemical nature of the lipid, ΔG° of partitioning remained in the range of −27 kJ/mol lipid in the gel phase and of −30 kJ/mol lipid in the fluid phase. This small difference cannot account for the observed phase-dependent differences in solubilization. Such virtually constant ΔG° occurred as a result of the compensation of enthalpic and entropic components, which varied with both temperature and lipid composition. Consequently, the observed different susceptibilities to solubilization cannot be attributed to differential binding but to further events in the solubilization process, e.g., bilayer saturability by detergent or propensity to form lipid-detergent mixed micelles. The data here shed light on the relatively unexplored early stages of membrane solubilization and open new ways to understand the phenomenon of membrane resistance toward detergent solubilization.

INTRODUCTION

Detergent solubilization of membrane proteins has been for many years, and will probably continue to be, a major tool in cell membrane investigation (1–3). The so-called ''raft hypothesis'' of transient microdomains in cell membranes (4) has been interpreted, often erroneously, in terms of ''rafts'' constituting detergent-resistant membrane domains (5). Raft studies have elicited a renewed interest in the mechanisms of membrane solubilization, and particularly in the solubilization of sphingolipids, which would be a major component of ''rafts'' (4).

A number of studies from this and other laboratories have shown that, in principle, bilayers formed by sphingolipids are easier, rather than more difficult, to solubilize than homologous glycerophospholipid membranes (6–8). In addition, since ''rafts'' are supposed to be more resistant to detergent solubilization at 4°C than at 37°C, a series of systematic studies of the effect of temperature on solubilization have been performed with the overall result that, for lipids in the fluid state, solubilization requires less detergent at lower temperatures (5,7–10), whereas, in the gel state, solubilization at lower temperatures requires more detergent (11). It should also be noted that ''rafts'' are supposed to exist in a particular form of the fluid phase, the so-called ''liquid-ordered phase'' (4). It has been shown that lipids in the liquid-ordered phase require more detergent for their solubilization than those in the liquid-disordered, commonly called fluid, phase (12).

As systematized by Helenius and Simons (2), the process of membrane solubilization by detergents can be divided into three stages, corresponding respectively to detergent partitioning into membranes (stage I), transition from detergent-saturated bilayer to lipid-detergent mixed micelles (stage II), and enrichment in detergent of the mixed micelles (stage III). The experiments mentioned above were focused primarily on the transition from stage I to stage II and/or the transition from stage II to stage III (see, e.g., Partearroyo et al. (13)). Stage I in itself has been comparatively less studied, partly because of the difficulties of measuring detergent binding in the absence of other phenomena. This difficulty has been overcome with the use of isothermal titration calorimetry (ITC).

This technique has been abundantly applied to the study of membrane-detergent interactions, but again focusing mainly on the solubilization step; see Heerklotz et al. (14), Keller et al. (15), Opatowski et al. (16), Wenk and Seelig (17), and Ollila and Slotte (18). These authors have established ITC as the tool of choice for the study of detergent partitioning into bilayers. However, a systematic study of partitioning along a wide range of temperatures, including a phase transition, was not available. In consequence this study was undertaken with the aim of studying, primarily by ITC but also with other techniques, the partitioning of Triton X-100 (Triton) into egg

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Address reprint requests to Félix M. Goñi, Fax: 34-94-601-33-60; E-mail: felix.goni@ehu.es.

Cristina Arnulphi's permanent address is Facultad de Matemática, Astronomía y Física, Universidad Nacional de Córdoba, Ciudad Universitaria, 5000 Córdoba, Argentina.

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sphingomyelin (SM), palmitoyl sphingomyelin (pSM), or dipalmitoylphosphatidylcholine (DPPC) bilayers. All three lipids exhibit a gel-fluid phase transition at $T \approx 38$–$41°C$. Measurements were carried out in the 5–55°C temperature range, under conditions in which the detergent caused no macroscopic effects on membrane structure, i.e., lipid/detergent mol ratios ≥ 10. Our results show that, through the compensation of enthalpic and entropic processes, $\Delta G°$ of detergent partitioning happens to be always negative and essentially invariant with temperature or type of lipid.

MATERIALS AND METHODS

Vesicle preparation

pSM, egg yolk SM (85% *N*-palmitoyl), and synthetic DPPC (purity >99%) were purchased from Avanti Polar Lipids (Alabaster, AL). For liposome preparation, phospholipids were dissolved in chloroform/methanol (2:1, v/v), and the mixture was evaporated to dryness under a stream of nitrogen. Traces of solvent were removed by evacuating the samples under high vacuum for at least 2 h. The samples were hydrated at 45°C in 20 mM PIPES (1,4-piperazinediethanesulfonic acid), 150 mM NaCl, 1 mM EDTA, pH 7.4 buffer, helping dispersion by stirring with a glass rod. To ensure homogeneous dispersions the hydrated samples were extruded between two syringes through a narrow tubing (0.5 mm internal diameter, 10 cm long) 100 times at 45°C. The solution was frozen in liquid nitrogen and thawed at 45°C 10 times. Large unilamellar vesicles (LUVs) were prepared by the extrusion method (19), using polycarbonate filters with a pore size of 0.1 μm (Nuclepore, Pleasanton, CA). Vesicle sizes were determined by dynamic light scattering using a Malvern Zetasizer instrument (Malvern, UK). The average vesicle diameter was 90–100 nm.

The nonionic surfactant Triton X-100 was purchased from Sigma (St. Louis, MO). Purity was >99%. Triton was diluted in the same PIPES buffer to a final concentration of 50–100 μM. The critical micellar concentration of Triton X-100 in our buffer, according to the method of Kaschny and Goñi (20), was 0.24 mM at 22°C.

DSC

To examine the thermotropic gel-fluid transition of the samples used in this study, DSC experiments were performed with an MC-2 high-sensitivity scanning calorimeter from MicroCal (Northampton, MA). The data were analyzed using Origin (Microcal) software provided by the calorimeter. SM or DPPC vesicles were incubated with Triton at different lipid/detergent mol ratios for 2 h to achieve equilibrium before calorimetric analysis.

Infrared spectroscopy

Infrared (IR) spectra were recorded in a Bruker Tensor 27 (Bruker Optics, Rheinstetten, Germany) spectrometer equipped with a mercury-cadmium-telluride detector using a demountable Peltier liquid cell (Biotools, Edmonton, Alberta, Canada) with excavated calcium fluoride windows, 25 μm optical path. Opus 5.0 software from Bruker Optics was used for data acquisition. Typically, 153 scans were collected at a nominal resolution of 2 cm^{-1} and averaged after each minute for each background and spectrum. Thermal studies were carried out by a continuous heating method. Samples were heated in the interval 10–60°C at 1°C/min in 50 min series, obtaining one averaged spectra per minute and degree.

Fluorescence spectroscopy

The generalized polarization (GP) of Laurdan was measured in a SLM-Aminco 8100 spectrofluorometer, equipped with thermoregulated cell holders (Aminco International, Lake Forest, CA). The excitation GP_{EX} parameter was calculated according to

$$GP_{EX} = (I_{440} - I_{490})/(I_{440} + I_{490}),$$

where I_{440} and I_{490} are the emission intensities obtained at 440 and 490 nm, respectively, exciting at 360 nm. The final probe/lipid molar ratio was 1:1000.

^{31}P-NMR spectroscopy

^{31}P-NMR spectra were recorded in a Bruker AV500 spectrometer operating at 500 MHz for protons (202.4 MHz for ^{31}P). The final phospholipid concentration was 100 mM. Spectral parameters were 45° pulses (11.5 μs), pulse interval of 1 s, sweep width of 30 kHz, and full proton decoupling. Two thousand free induction decays were routinely accumulated from each sample; the spectra were plotted with a line broadening of 40 Hz. Samples were equilibrated for at least 10 min at each temperature before data acquisition.

Isothermal titration calorimetry

The enthalpy change upon partitioning of Triton into SM LUVs could be measured with high-sensitivity ITC. ITC was performed using a model VP-ITC high sensitivity titration calorimeter (MicroCal, Northampton, MA). In this study the calorimetric cell was filled with Triton at 50–100 μM, i.e., well below its critical micellar concentration. Lipid vesicles at a 6–13 mM lipid concentration were injected into the cell (1.43 mL) in 3–10 μL steps, i.e., leading to a 100–200-fold dilution of lipid vesicles. To minimize the contribution of dilution to the heat of partitioning, both the lipid vesicles and the detergent solution were prepared in the same buffer. Detergent solutions were degassed under vacuum immediately before use. Typically, the injections were made at 10 min intervals and at 2 s/μL. Constant stirring speed of 290 rpm was maintained during the experiment to ensure proper mixing after each injection. Dilution heats of lipid vesicles into the buffer were determined in separate experiments and subtracted from experimental heats of binding. At each lipid injection, free detergent monomers partitioned into the bilayer membrane and the corresponding heat of reaction was measured. The heat of reaction became smaller as less and less surfactant remained free in solution. The integration of each calorimetric peak yields a heat of reaction. These heats were plotted versus the lipid concentration. The obtained isotherm was used to determine the thermodynamic parameters of partitioning (21,22).

A model of partition equilibrium of the detergent molecules between aqueous phase and the membrane is proposed for the interaction. In this model it is assumed that Henry's law applies, i.e., there is a linear relationship between the concentration of surfactant free in solution C_{TX}^{W} and the concentration bound to the membraneC_{Tx}^{b} (23). If we denote by C_L the total lipid concentration, the affinity of the membrane for TX-100 is quantified by the molar partition constant K

$$K \equiv \frac{C_{TX}^{b}}{C_L C_{TX}^{w}}. \quad (1)$$

Note that K is the reciprocal of the lipid concentration required to bind 50% of the detergent.

The fit of the experimental data to the binding model gives the molar partition constant K and the enthalpy change ΔH. Through these values the standard free energy $\Delta G°$ and the standard entropy change $\Delta S°$ can be calculated:

$$\Delta G° = -RT \ln K \quad (2)$$

$$\Delta G° = \Delta H - T\Delta S°. \quad (3)$$

(Strictly speaking, $\Delta G°$ and $\Delta S°$ in our case refer to standard conditions except that temperature is not always 25°C. Also $\Delta G°$ should be computed

from ΔH° and not from ΔH, but ΔH is the measured value in our case and it varies little with concentration so that we can assume $\Delta H \approx \Delta H^\circ$.)

The heat capacity change ΔC_p for the transfer of detergent monomers into vesicles was calculated from the temperature dependence of the ΔH values

$$\Delta C_p \approx \Delta\Delta H/\Delta T. \quad (4)$$

The experimental data were analyzed using the Origin software as provided by Microcal. For the fitting of the data to the partitioning model the PartiRel program, developed by Heerklotz et al. (14), was used with permission of the authors.

Statistics

All data are given as average values ± SE of three measurements obtained with different vesicle preparations. When required, statistical significance was measured with Student's *t*-test.

RESULTS

Macroscopic effects of Triton X-100 on membranes, i.e., permeabilization, fusion, and/or solubilization, occur at lipid/detergent molar ratios below 2 (24–27). To examine the effects of detergent partitioning uninfluenced by other phenomena, we used lipid/detergent molar ratios ≥10. Note that throughout this article ''total'' rather than ''effective'' (i.e., in the bilayer) lipid/detergent ratios are given. However, considering the high lipid/water partition coefficient of Triton X-100 (27), it can be safely assumed that total and effective lipid/detergent ratios are the same within experimental error (except in the early stages of the titration experiments, in which small amounts of lipid are added to a detergent suspension).

Direct measurements of detergent binding to phospholipid bilayers have been carried out using ITC whose data constitute the core of this study. However, ITC results can only be properly interpreted if a system representing the endpoint of titration, i.e., bilayers with a 10:1 (mol ratio) phospholipid/detergent ratio, is first described. Such a description of a lipid bilayer containing a nonsolubilizing proportion of Triton X-100 has been achieved using DSC, IR spectroscopy, and Laurdan GP. Thus the sections below first present the data obtained with those three techniques then describe the ITC experiments.

DSC

Pure egg SM exhibits a relatively narrow gel-fluid transition by DSC, centered at 39.4 ± 0.16°C, width at half-height 2.9°C ± 0.18°C, ΔH = 5.4 ± 0.56 kcal/mol, in agreement with previous studies (28,29). Triton causes a dose-dependent shift of the transition to lower temperatures and a widening of the transition temperature range (Fig. 1 *A*). The midpoint transition temperature goes down to 37.1°C ± 0.07°C for the 10:1 (mol ratio) SM/Triton mixture, and the corresponding width at half-height and ΔH are, respectively, of 4.56°C ± 0.06°C and 3.4°C ± 0.07°C kcal/mol. A comparable study with pSM provided very similar results (Supplementary Material Fig. S1), except that the phase transition of the pure lipid was centered at 40.8°C ± 0.04°C and shifted to 39.8°C ± 0.01°C in the presence of detergent (10:1 mol ratio). Width at mid-height increased from 2.2°C ± 0.01°C to 3.2°C ± 0.01°C, and ΔH decreased from 11.8°C ± 0.11°C kcal/mol to 7.6°C ± 0.08°C kcal/mol. The downshift in transition temperature indicates an overall fluidizing effect of Triton, which had been equally observed in DPPC/Triton mixtures (30) and is also detected for the range of lipid/detergent ratios used in this study (Fig. 1 *B*). In our case, T_m, the width at half-height, and ΔH are, respectively, 41.5°C ± 0.01°C, 0.76°C ± 0.001°C, and 7.1°C ± 0.01°C kcal/mol for the pure lipid and 40.8°C ± 0.04°C, 2.2°C ± 0.002°C, and 6.6°C ± 0.01°C kcal/mol for the 10:1 DPPC/Triton mixture.

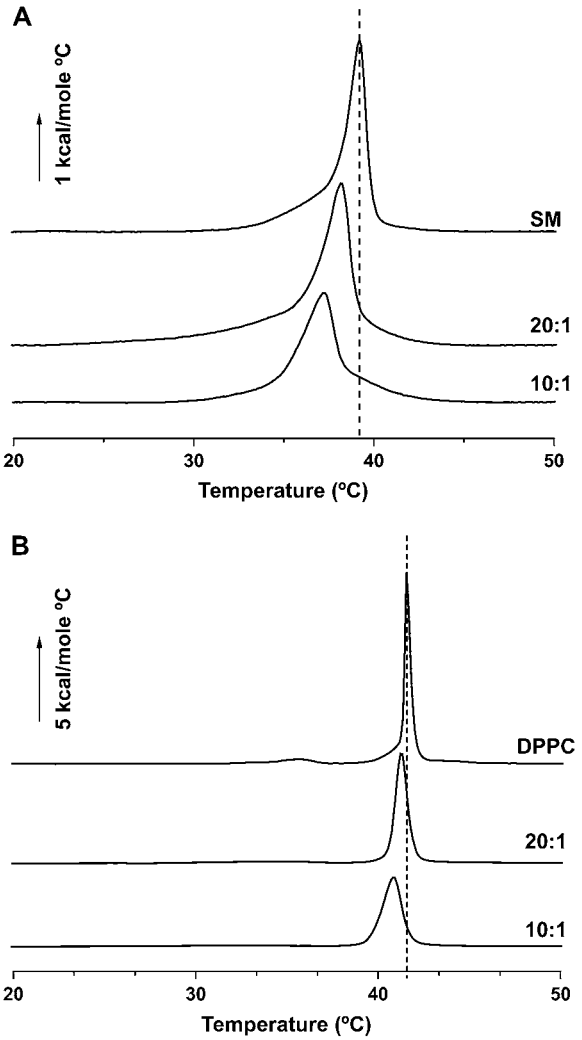

FIGURE 1 DSC thermograms corresponding to the gel-fluid transition of phospholipid vesicles incubated with increasing amounts of Triton. (*A*) SM bilayers. (*B*) DPPC bilayers. When appropriate, lipid/detergent mol ratios are indicated for each thermogram. Third heating scan.

Infrared spectroscopy

Triton causes significant changes in the IR spectrum of egg SM at concentrations well below those causing solubilization. Fig. S2 (Supplementary Material) shows representative regions of the SM spectrum in the presence and absence of Triton at a 20:1 lipid/detergent ratio and 45°C. The methyl and methylene C-H stretching region is plotted in Fig. S2 *a*. Triton causes a spectral shift to higher wavenumbers that is usually interpreted as an increase in *gauche* rotamers, i.e., a disordering of the hydrocarbon chains (31,32).

When the position of the SM asymmetric C-H stretching vibration band is plotted as a function of temperature, in the 10–60°C range (Supplementary Material Fig. S3), an abrupt increase is observed centered at 40°C, i.e., at the gel-fluid transition, when the proportion of *gauche* rotamers is greatly increased. The corresponding data for a SM/Triton 20:1 mixture, also plotted in Fig. S3, show two effects, namely, i), a shift in band position of ~1 wavenumber irrespective of temperature; and ii), a downshift in transition temperature of ~2°C, in agreement with the DSC data.

The amide group of SM gives rise to a distinctive band, centered at $\approx 1650\ cm^{-1}$. Subsolubilizing concentrations of Triton also have an effect on this band, which reports on the bilayer region at the lipid-water interface (Supplementary Material Fig. S2 *b*). The detergent downshifts the amide band by an average of 2.5 wavenumbers, irrespective of temperature, and increases the width at half-height by ~1.2 wavenumbers, also irrespective of temperature (within 10 and 60°C) (spectra not shown). The shift in band position suggests either a reorientation of the amide band or a reorganization of the H-bond network in which the amide group is involved (33). The increase in width is usually interpreted as an increase in the librational motion of the amide group (32,34). Thus low concentrations of Triton appear to cause significant changes in the conformation and motility of the SM portion at the lipid-water interfaces.

Laurdan generalized polarization

Laurdan is well known as a polarity-sensitive fluorescent probe in membranes (35). The so-called ''generalized polarization'' (GP) function has been developed as a convenient measurement of the probe environmental polarity (36). GP possesses all the properties of the classical fluorescence polarization, e.g., it can be used to mark, as an abrupt decrease, the gel-fluid transition of phospholipids in bilayers. The temperature variation of Laurdan GP in SM and SM/Triton (20:1 mol ratio) bilayers is plotted in Fig. 2 *A*. Similar measurements carried out with pSM are shown in Supplementary Material Fig. S4. In both cases, the main detergent effect is the increase in Laurdan polarization, particularly below the main transition temperature of SM. The fluorescent naphthalene moiety of Laurdan is located at the level of the hydrocarbon methylene groups close to the interface. The

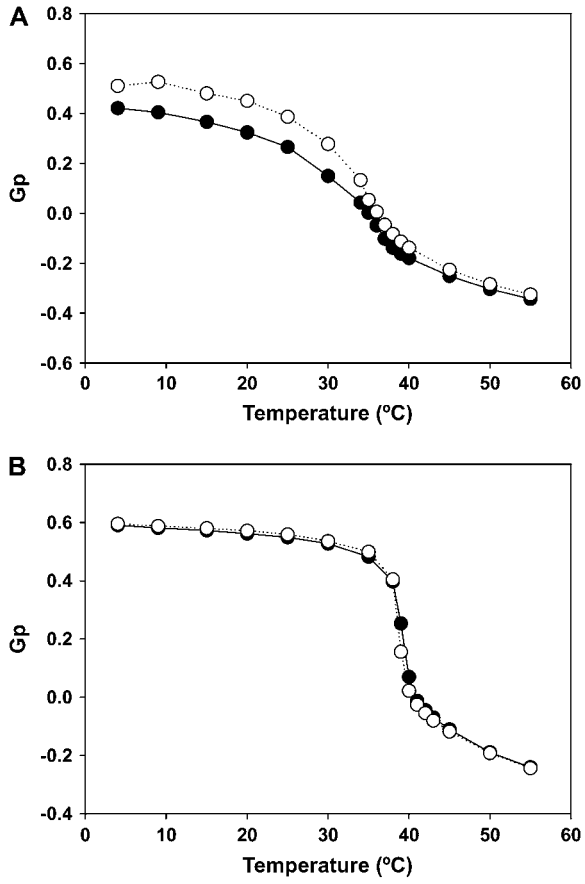

FIGURE 2 Temperature variation of the GP of the fluorescent probe Laurdan embedded in phospholipid bilayers. (*A*) SM bilayers. (*B*) DPPC bilayers. (●) Pure phospholipid. (○) Phospholipid/Triton (20:1 mol ratio). Average values of three independent experiments ± SE. The standard errors are smaller than the size of the symbols.

detergent-induced increase in Laurdan polarization may be an indication of a lower penetration of water at the membrane interface. It is possible that some water molecules are being replaced by detergent. This would also explain the changes in the H-bond network detected by the IR amide band (Supplementary Material Fig. S1 *b*).

Previous experiments from this laboratory (30) have shown that Triton binding to bilayers composed of saturated phosphatidylcholines has qualitatively similar effects as binding to SM bilayers, judging from DSC and IR measurements: Triton lowers the transition temperature of saturated PC bilayers (Fig. 1 *B*) (30,38) and disorders the lipid chains (30). Laurdan GP provides a different point of view (Fig. 2): the absolute values of polarization are higher for DPPC than for SM at all temperatures and particularly in the gel phase. In addition, Triton does not cause any significant change in GP when added to DPPC bilayers (Fig. 2 *B*). Thus SM bilayers appear to be more accessible to water/detergent molecules than DPPC. The width of the phase transition for DPPC is smaller than that of SM, as seen both by Laurdan GP (Fig. 2) and by DSC (Fig. 1).

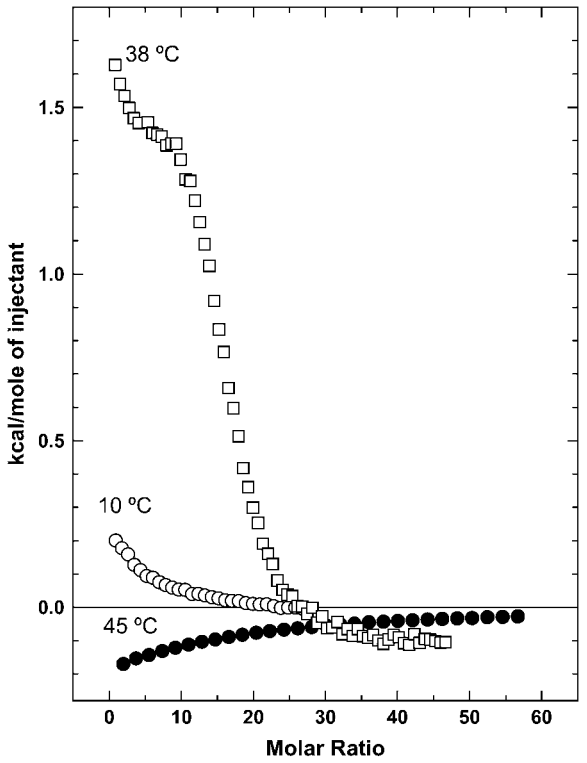

FIGURE 3 Comparison of representative ITC isotherms for the interaction of Triton with SM at different lipid phase states: (○) gel phase (10°C), (●) fluid phase (45°C), and (□) within the lipid phase transition (38°C).

Isothermal titration calorimetry

Triton binding to LUVs composed of pure egg SM was measured by ITC along a wide range of temperatures. In these experiments, small (3–10 μl) volumes of SM LUVs (6–13 mM) are repeatedly injected into a large (1.43 ml) volume of Triton at a concentration (50–100 μM) well below the detergent critical micellar concentration. The crude experimental results (Supplementary Material Fig. S5, *a–c*) are converted into plots of heat exchanged/mol SM as a function of lipid/detergent mol ratio (Fig. S5, *d–f*). Exchanged heat/mol SM is maximum for the first injection of vesicles and tends toward zero when no more detergent exists free in solution. The latter situation occurs at 30–60 lipid/detergent mol ratios. In principle, the measured heat contains, in addition to the heat of partitioning, a component that arises from vesicle dilution. However the latter was measured independently and found to be negligible ($\leq$5% of total heat exchange) but still routinely subtracted.

Theoretical curves representing several partitioning models can be fitted to the plots of exchanged heat/mol SM versus lipid/detergent ratio. Although a model of ligand binding by a macromolecule with n identical sites fits some of our results, the best fit that could be found for the largest number of curves followed a partition model, which has been used by other authors with this aim (37). This model is based on the assumption of a linear relationship between the concentration of surfactant free in solution and the concentration of membrane-bound surfactant (23). In Fig. S5, data retrieved at 10°C and 45°C could be fitted to the partition model (see continuous curve in panels *d* and *f*) but not data obtained at 38°C (panel *e*). In general, fitting to a simple model was not

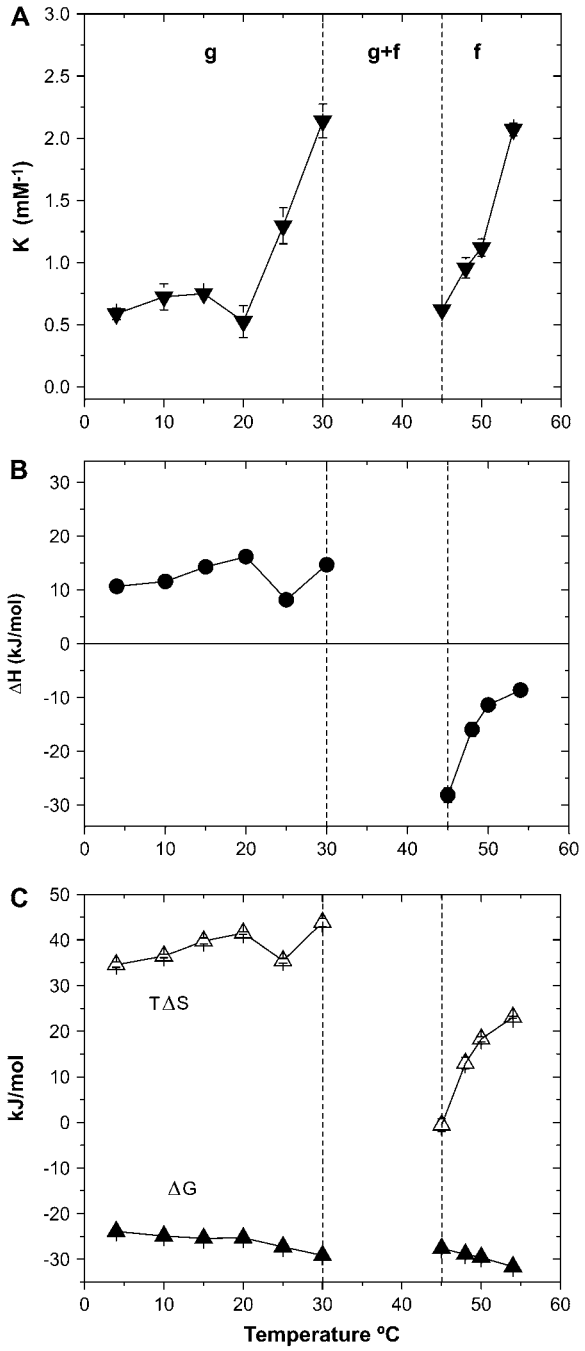

FIGURE 4 Thermodynamic parameters for the interaction of Triton with SM vesicles studied as a function of temperature. (*A*) Partition constant, *K*. (*B*) Partition enthalpy ΔH. (*C*) Free energy and entropy change. Gel (*g*) and fluid (*f*) phases are indicated in the appropriate temperature ranges. Average values if three independent experiments ± SE. The standard errors are often smaller than the size of the symbols.

possible at temperatures close to the gel-fluid transition temperature of SM (see below). In all other cases, the partition model curves consistently fitted the experimental values with $r^2 > 0.99$.

The temperatures at which the experiments in Fig. S5 have been performed correspond to SM and SM/Triton mixtures in the gel state (10°C), in the fluid state (45°C), and within the phase transition (38°C) (see Fig. 1 *A*). Comparison of the calorimetric data can be seen in Fig. 3. Detergent partitioning in the gel state is endothermic, but it is exothermic in the fluid state. During the phase transition, detergent partitioning can be endo- or exothermic, depending on the lipid/detergent mol ratios, but the endothermic component is extremely large as compared to that found in both the gel and fluid situations. This large heat exchange, together with the fact that at the phase transition calorimetric data cannot be fitted to the partition model, suggests that phenomena other than partitioning are taking place under these conditions. The possibility that even at these high lipid/detergent ratios some solubilization was taking place at the transition temperature was examined by ^{31}P-NMR. Spectra of SM/Triton 20:1 and 10:1 mixtures were recorded in the 5–55°C *T* range. Under these conditions, the spectra showed invariably the line shape characteristic of phospholipids in the bilayer form: a narrow isotropic signal that could arise from SM/detergent mixed micelles was never observed (Supplementary Material Fig. S6). Thus partial solubilization under our conditions could be excluded.

An alternative reasonable explanation is that, at temperatures close to the gel-phase transition temperature, Triton is inducing an isothermal phase transition, i.e., moving molecules (or domains) from the gel to the fluid phase. This would be in agreement with the fluidifying and disordering properties of Triton, seen both by DSC and by IR spectroscopy (Fig. 1, and Figs. S2 and S3 in the Supplementary Material). The heat exchange measured at 38°C for the first vesicle injection is of ~1.7 kcal/mol SM; this is of the same order of magnitude as the ΔH of the SM phase transition, measured by DSC, of 5.3 kcal/mol (see above). Thus it is suggested that, at temperatures close to the gel-fluid thermotropic transition temperature of SM, the measured heat exchange corresponds largely to a phase transition heat, heat of partitioning being only a minor component.

Fitting the calorimetric results to a partition model allows the calculation of a lipid/water partition constant for the detergent, *K*, and from it the standard Gibbs free energy of binding, $\Delta G°$. Binding enthalpies ΔH are directly obtained from the calorimetric results, and from $\Delta G°$ and ΔH, $T\Delta S°$ is readily computed. Values for these parameters of Triton binding to SM in the 4–55°C range are shown in Fig. 4, with the exception of the phase transition region (30–45°C) for the reasons discussed above. In the gel state, *K* remains constant up to 20°C, then it increases by ~4-fold, in principle suggesting more favorable partitioning between 20°C and 30°C (Fig. 4 *A*). The partition enthalpy ΔH remains virtually

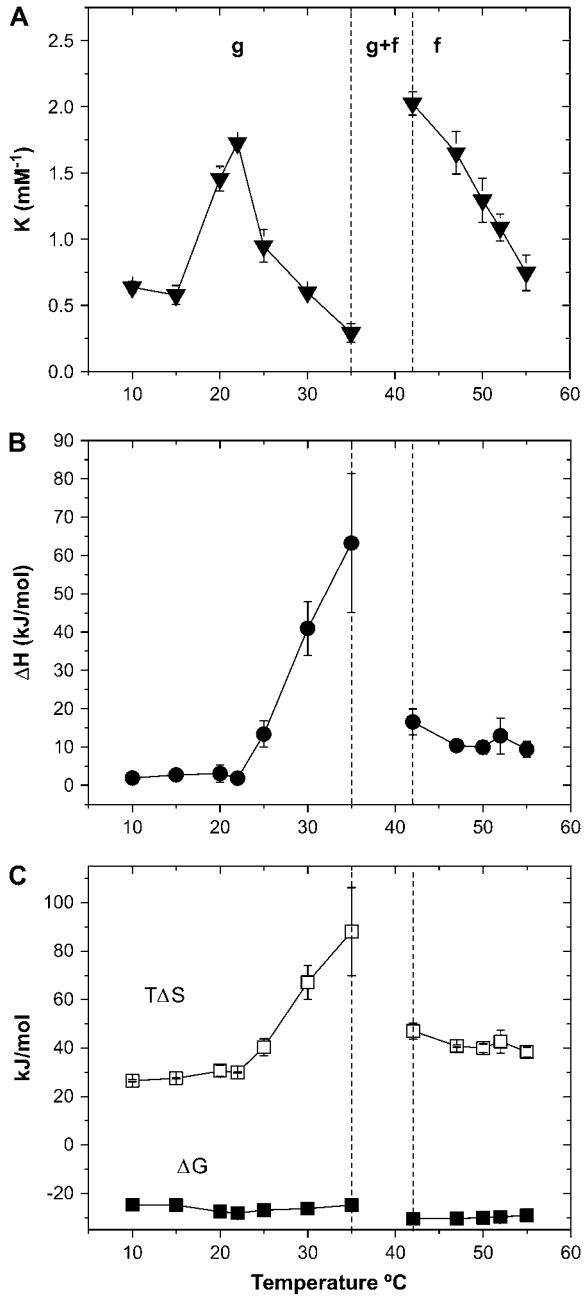

FIGURE 5 Thermodynamic parameters of the partition of Triton into DPPC membranes as a function of temperature. (*A*) Partition constant, *K*. (*B*) Partition enthalpy, ΔH. (*C*) Entropy and free energy change. Gel (*g*) and fluid (*f*) phases are indicated in the appropriate temperature ranges. Average values of three independent experiments ± SE. The standard errors are sometimes smaller than the size of the symbols.

constant in the whole gel phase *T* interval (Fig. 4 *B*), thus $\Delta G°$ decreases though not markedly above 20°C (Fig. 4 *C*).

Note that, because of the logarithmic relationship between $\Delta G°$ and *K*, a fourfold increase in *K* will only change $\Delta G°$ by $RT \ln 4$, i.e., ≈ 3.4 kJ/mol. The entropic component $T\Delta S°$ is quantitatively important in this temperature range, suggesting

that in the gel state the highly ordered lipid is being disordered by Triton (see IR results in Supplementary Material Figs. S2 and S3) and that this disordering joins the increase in ΔS° arising from Triton dehydration to make the partitioning thermodynamically favorable. The increase in the partition constant between 20°C and 30°C may be related to the observed decrease in Laurdan GP, which occurs in this temperature range for SM but not for DPPC (Fig. 2). No other technique in this study detects any change in the SM bilayer in this temperature range. The decrease in Laurdan GP may be monitoring a higher penetration of water in the membrane interface, and this in turn may be facilitating detergent partition.

In the fluid phase just above the transition temperature, i.e., at 45°C, K has returned to the low values ($\approx$0.6 mM^{-1}) that were found below 20°C (Fig. 4 *A*), but increasing temperatures from 45°C to 55°C cause a new rise in K, so that at the latter temperature K has reached again its value at 30°C. Thus K appears to have a general trend of increasing with temperature, with a discontinuity at the phase transition. The data are compatible with Triton partitioning, within a given lipid phase, preferentially at higher temperatures.

The binding enthalpy ΔH becomes negative in the fluid state, i.e., binding is exothermic under these conditions, although it becomes less so with temperature (Fig. 4 *B*). Similar sign changes in the ΔH of detergent binding have been observed in other systems (15,16). ΔG° is somewhat lower in the fluid than in the gel phase. The average ΔG° values are -29.5 ± 1.48 kJ/mol in the fluid phase versus -27.3 ± 2.35 kJ/mol in the gel phase ($n = 5$, $p < 0.05$). This rather small difference may, however, explain a preference for Triton to partition in the fluid phase, hence its tendency to lower the gel-fluid transition temperature (Fig. 1 and Fig. S3 of the Supplementary Material). The small ΔG° change above and below the transition temperature, despite the abrupt change in ΔH, is due to entropic compensation. The entropic component is less important in the fluid than it was in the gel phase (Fig. 4 *C*), perhaps because Triton is not causing major disordering of the already disordered chains under these conditions.

ITC measurements of Triton partition into pSM bilayers were performed at 10°C and 50°C, i.e., in the gel and fluid phases (see Supplementary Material Fig. S1). The results are summarized in Table 1, in parallel with the corresponding values for egg SM. In general rather similar values are obtained with both lipids. K is somewhat lower for pSM than for egg SM, and ΔH is slightly more positive at 10°C and slightly more negative at 50°C. However when partitioning is expressed in terms of ΔG, the differences are too small (<1.5kJ/mol) to influence detergent binding in any detectable way.

ITC data for Triton partitioning into DPPC bilayers, in the form of LUVs, are summarized in Fig. 5, and the difference with egg SM can be largely explained on the basis of the structural differences just mentioned. Well below the phase transition, partitioning is almost isocaloric ($\Delta H \approx 0$) (Fig. 5 *B*). However the partition constant K varies within the same range of values as it does for SM, i.e., 0.5–2.0 mM^{-1} (Fig. 5 *A*). The result is that, in the region below 22°C, partition thermodynamics is dominated by an entropic component due to both Triton dehydration and disordering the quasicrystalline structure of the DPPC gel phase at these temperatures. At higher T, always within the gel phase, ΔH increases the endothermic process revealing once again the breakdown of intermolecular (van der Waals?) associations that accompanies insertion. In the fluid phase both the entropic and enthalpic components contribute to the negative ΔG°, which, as in the case of SM, varies little with temperature although there is an average of -3.4 kJ/mol when going from the gel to the fluid phase (Fig. 5 *C*).

The main difference between the egg SM and DPPC data refers to the temperature variation of the partition constant K. It tends to decrease with T in the case of DPPC (Fig. 5 *A*), whereas the opposite is seen in SM (Fig. 4 *A*). The available data do not provide a simple explanation for the difference although the T-dependent weakening of the H-bonds (39) may explain the increase in K in SM bilayers.

Finally, it should be noted that ΔG° of binding is not only virtually constant in the temperature range under study, but it is also equal, within experimental error, in pSM, egg SM, and DPPC (Fig. 6). It is interesting that the enthalpic and entropic components are compensated differently in each case to give rise to a situation in which Triton binding to bilayers is equally favored, irrespective of temperature or the chemical nature of the lipid.

DISCUSSION

Perhaps the most remarkable result in this study is the observation that ΔG° of detergent binding to lipid bilayers is virtually invariant with temperature and with the lipid composition, SM, or DPPC of the membranes. This invariant ΔG° does not reflect uniform modes of detergent-lipid interactions but is rather the result of different compensatory enthalpic/entropic phenomena. Our observations are better

TABLE 1 Comparison of the thermodynamic parameters of Triton X-100 partition into pSM and egg SM bilayers, as computed from ITC measurements; average values ± SE ($n = 3$)

	K (mM^{-1})		ΔG (kJ/mol)		ΔH (kJ/mol)		$T\Delta S$ (kJ/mol)	
	10°C	50°C	10°C	50°C	10°C	50°C	10°C	50°C
Egg SM	0.72 ± 0.105	1.12 ± 0.069	−24.9 ± 0.06	−29.5 ± 0.02	11.5 ± 0.38	−11.4 ± 0.57	36.5 ± 0.38	18.2 ± 0.57
pSM	0.39 ± 0.112	0.68 ± 0.037	−23.5 ± 0.01	−28.3 ± 0.01	15.9 ± 2.20	−21.5 ± 2.19	39.4 ± 0.38	7.1 ± 2.2

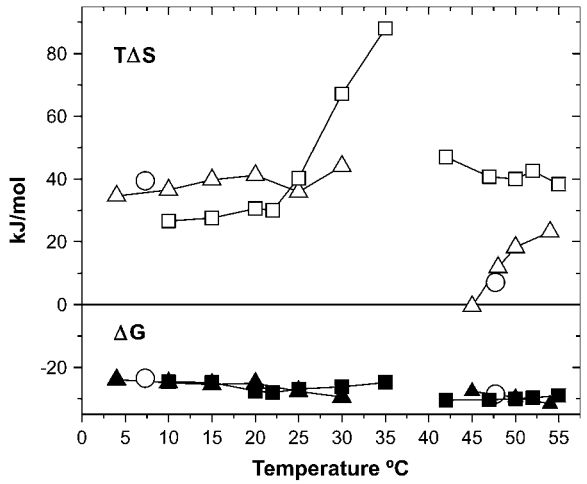

FIGURE 6 Comparative values of $T\Delta S$ and ΔG for the interaction of Triton with SM and DPPC membranes. (■) SM. (●) DPPC. (○) pSM. Average values of three independent experiments. SM and DPPC data are redrawn from Figs. 4 and 5, except that error bars have been omitted for the sake of clarity. For pSM data, the standard error is smaller than the size of the symbols.

understood in the framework of i), previous studies, ii), the molecular interactions involved, and iii), the practical consequences.

Previous studies

The observation of Triton partitioning at very high ($\geq$10) lipid/detergent ratios (''stage I'' of Helenius and Simons (2)) has been possible only with the use of ITC. Seelig and co-workers (37,41) and Blume and co-workers (42) were among the first to apply this technique in the study of membrane-amphiphile interactions. Keller et al. (15) measured the binding (partitioning) of octylglucoside to phosphatidylcholine bilayers. As in our case, they observed that the process is endothermic at low temperature (27°C) and exothermic at high temperature (70°C). ΔH at 27°C was 3.04 kcal/mol, in the same order of magnitude as those found in this study at that temperature (Figs. 4 *B* and 5 *B*). The change in sign of ΔH was also described by Opatowski et al. (16) for the partitioning of octylglucoside into egg phosphatidylcholine bilayers although in their protocol the extraction of surfactant from bilayers to solvent was measured; consequently they observe that below 40°C the process is exothermic and becomes endothermic at higher temperatures. (Occasional experiments in the ''release mode'' (43) carried out in our laboratory with the SM/Triton system provided ''mirror-image'' results compared to those performed in the partition mode.) Both Keller et al. (15) and Opatowski et al. (16) observed a temperature-dependent change in ΔH sign in the absence of a phase transition. Thus may be our comparable observations with SM (Fig. 4 *B*) are in fact independent of the lipid phase transition.

In a more recent study, Ollila and Slotte (16) studied, among other aspects, the partitioning of Triton X-100 into egg SM bilayers at 25°C. Their results are in good agreement with ours (Fig. 4), particularly considering the natural origin of the lipid. They measured a partitioning constant $K = 2$ mM^{-1} and enthalpy change $\Delta H = 1.5$ kcal/mol SM, and obtained a $\Delta G^\circ = -4.5$ kcal/mol SM. Other authors had also found similar values for K, e.g., Pantaler et al. (44) measured $K = 2.4$ mM^{-1} for Triton partitioning into erythrocyte membranes; and the results by Heerklotz et al. (45) for Triton transfer from buffer to 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC) membranes at ~20°C correspond very well to our data for DPPC at 50°C, i.e., when both bilayers are in a comparable state of fluidity.

Molecular interactions

The molecular interactions that result in the partition of Triton into phospholipid bilayers can be dissected into three groups of concurrent effects, namely, the hydrophobic effect, temperature effects, and structural effects. The importance of the hydrophobic effect on the stability of the lipid bilayer and its relevance in explaining membrane solubilization by detergents has been pointed out repeatedly (1,16,22,46–50). As stated by Ollila and Slotte (16), ''the reduction of the intermolecular hydrogen bonding capacity of water, caused by the hydrophobic moieties of the detergent molecules, forces the detergent molecules to be distributed between the hydrophobic environment in the membranes and the bulk water. [...] the removal of the hydrophobic moieties from the polar phase to the bulk hydrocarbon in the lipid membrane [...] increases the overall entropy of the system and makes partitioning possible''. This is certainly the situation in our case. $T\Delta S$ is consistently positive so that even if partitioning is under most conditions endothermic (Figs. 4 and 5), ΔG is negative and the process is energetically favorable.

For Triton partitioning into fluid SM, the process is exothermic (Fig. 4 *B*). Under these conditions, the entropic contribution to the spontaneous process is relatively small. This situation has been described as ''nonclassical hydrophobic effect'' in membrane binding equilibria (37). The enthalpy-entropy compensation is a common phenomenon in biological interactions. It has been observed as well in ITC studies in peptide- and protein-lipid interactions in membranes (51–53). Enthalpy-entropy compensation has been interpreted as the result of conformational fluctuations of the system in the boundaries of the interaction site (54).

Partition phenomena dominated by the hydrophobic effect, e.g., the insertion of some peptides into lipid bilayers, have been described in which ΔC_P is invariably negative. These studies have been performed with phosphatidylcholine bilayers in the fluid state. Triton partition into fluid DPPC bilayers also has a negative ΔC_P. From the data in Fig. 5, a value of $\Delta C_P = -95$ cal/K $\times$ mol can be obtained, in good agreement with data for detergents (15) and peptides

(see above references). However, ΔC_P is positive in the case of fluid SM (Fig. 4) and in the gel phase for both SM and DPPC (Figs. 4 and 5). Once again H-bonding may influence the results with SM, and partition to the gel state may obey different rules. Thus, a negative ΔC_P should not be considered as the universal signature for partition phenomena governed by the hydrophobic effect. Rowe et al. (48), studying the membrane partitioning of *n*-alcohols, also found that although hydrophobic interactions played an important role in partitioning, the contributions from changes in the structure and thermodynamic state of the bilayer, including the interfacial region, could not be ignored.

Temperature effects are important in all cases because they influence thermal motion; but in the cases of SM and DPPC, they are in addition responsible for the gel-fluid thermotropic transitions.

The partition constant K described in this study is an association constant, and it is seen to decrease with temperature under most circumstances for the case of Triton/DPPC interaction (Fig. 5 *A*). The mole fraction partition coefficient, a parameter closely related to the mole ratio partition constant used in this study, also often decreases with temperature, and in fact it changes in this way for the partitioning of detergents into phosphatidylcholine bilayers (18). As mentioned above, temperature also has the obvious effect of causing the thermotropic gel-fluid transition with a concomitant abrupt increase in lipid order and fluidity, which cause clear discontinuities in K, ΔH, and $T\Delta S°$ (Figs. 4 and 5).

Finally, structural effects are mainly responsible for the observed differences between SM and DPPC. These two phospholipids are similar in many respects. However an important difference is that the interface moiety of SM contains a free OH group in the sphingosine chain and an amide group, both of which cooperate to give rise to a complex network of intermolecular H-bonds that do not occur in DPPC. The strong H-bonding properties of SM lead to considerable structural changes in the polar headgroup and interface regions (33). In particular, the H-bonding network leads to a strong ordering of the SM acyl chains. H-bonding, however, is very sensitive to temperature, becoming weaker at higher T (39). This could explain why in SM, opposite to what happens with DPPC, K values increase with T because in SM, partitioning of Triton appears to be hindered by the strong H-bonding, and this effect overcomes the temperature-dependent decrease in partitioning.

Consequences for membrane solubilization by detergents

Considerable experimental evidence exists showing that Triton solubilization of both DPPC and SM is temperature sensitive, e.g., DPPC is more easily solubilized at 47°C than at 55°C, and SM is more easily solubilized at 22°C than at 50°C (7,11). Also, egg SM is more easily solubilized (solubilization requires less detergent) than PC, both being in the fluid state and at similar temperatures (6,7). The origin of these differences is not known with certainty, but our results suggest that they cannot be explained by changes in $\Delta G°$ of detergent partitioning (Figs. 4 and 5), i.e., they cannot be attributed to different affinities for Triton. Rather, the differences may be due either to i), a different saturation capacity of the various bilayers at different temperatures so that saturation, or transition from stage I to stage II, would occur at a different lipid/detergent ratio; or ii), differences within the far less well understood stage II. These possibilities remain open to experimentation.

The observation that $\Delta G°$ is almost the same above and below the transition temperature supports the idea that it could also be similar during the thermotropic transition. In that case, $\Delta G°$ values obtained by interpolation could be combined with experimental (DSC) values of ΔH of the phase transition, thus allowing a more detailed study of the thermodynamic parameters of detergent binding during the phase transition. The small decrease in $\Delta G°$ of partitioning when going from the gel to the fluid phase is similar to the $\Delta\Delta G°$ found by Heerklotz et al. (45) between Triton partition into fluid-disordered POPC and fluid-ordered POPC/SM/cholesterol bilayers. This would explain, in our case, the ability of Triton to downshift the transition temperature and fluidize the bilayers. Triton preference for binding fluid-disordered regions would be compatible with Triton partitioning, in the gel, to disordered microdomains arising from thermal fluctuations. Clusters of detergent molecules would form under these conditions that would become more uniformly distributed above the transition temperature. Again this hypothesis is amenable to experimental testing. Note that Triton may not be unique in its tendency to accumulate in small fluid defects. Other ''foreign'' molecules, e.g., Laurdan, may do the same, and this would explain the ability of Laurdan (Fig. 2) to detect thermal effects at temperatures, e.g., 30°C, when DSC or IR, which report on the average lipid, fail to report any thermal changes.

SUPPLEMENTARY MATERIAL

To view all of the supplemental files associated with this article, visit www.biophysj.org.

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