Triton Promotes Domain Formation in Lipid Raft Mixtures

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ABSTRACT Biological membranes are supposed to contain functional domains (lipid rafts) made up in particular of sphingomyelin and cholesterol, glycolipids, and certain proteins. It is often assumed that the application of the detergent Triton at 4°C allows the isolation of these rafts as a detergent-resistant membrane fraction. The current study aims to clarify whether and how Triton changes the domain properties. To this end, temperature-dependent transitions in vesicles of an equimolar mixture of 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine, egg sphingomyelin, and cholesterol were monitored at different Triton concentrations by differential scanning calorimetry and pressure perturbation calorimetry. Transitions initiated by the addition of Triton to the lipid mixture were studied by isothermal titration calorimetry, and the structure was investigated by ^{31}P-NMR. The results are discussed in terms of liquid-disordered (ld) and -ordered (lo) bilayer and micellar (mic) phases, and the typical sequence encountered with increasing Triton content or decreasing temperature is ld, ld + lo, ld + lo + mic, and lo + mic. That means that addition of Triton may create ordered domains in a homogeneous fluid membrane, which are, in turn, Triton resistant upon subsequent membrane solubilization. Hence, detergent-resistant membranes should not be assumed to resemble biological rafts in size, structure, composition, or even existence. Functional rafts may not be steady phenomena; they might form, grow, cluster or break up, shrink, and vanish according to functional requirements, regulated by rather subtle changes in the activity of membrane disordering or ordering compounds.

INTRODUCTION

The fascinating idea of lipid rafts as functional domains in biological membranes (Brown and London, 1997; Simons and Ikonen, 1997; Jacobson and Dietrich, 1999) has directed enormous research efforts toward the molecular biology of membrane lipids. The term raft denotes relatively large and long-lived domains composed of specific lipid species such as sphingomyelin (SM), cholesterol (Cho), and glycolipids in biological membranes. They are suggested to accumulate certain membrane proteins (often anchored by saturated alkyl chains) that could, for example, greatly enhance signaling cascades or crucially interfere with many other functions (Simons and Ikonen, 1997). Single particle tracking (Dietrich et al., 2002), single dye tracing (Schutz et al., 2000), fluorescence microscopy (Radhakrishnan et al., 2000; Dietrich et al., 2001a,b), and fluorescence quenching (Ahmed et al., 1997; Schroeder et al., 1998; Wang and Silvius, 2001) are major tools for studying biomembrane and model membrane heterogeneity. Although physical evidence for rafts is increasing, a detailed understanding of the processes is still lacking. Recently, Simons and Toomre (2000) noted that ordered domains in biological membranes are initially very small and need to be clustered transiently to form the large rafts including many proteins required for typical raft functions.

By its nature and the history of its discovery, the concept of functional lipid rafts in biological membranes is intimately connected with a related concept, that of so-called detergent-resistant membranes (DRMs) (Yu et al., 1973; Schroeder et al., 1994; Patra et al., 1999; London and Brown, 2000). DRMs are phenomenologically defined. Application of the detergent Triton X-100 (TX) to cell membranes at 4°C leads to a coexistence of small micelles (detergent-soluble fraction, containing, e.g., unsaturated diacylphosphatidylcholine (PC) and certain proteins) and larger particles (DRMs, containing, e.g., SM, Cho, and supposedly raft-associated proteins) that can be separated by centrifugation.

Finally, a third concept, that of the liquid-ordered (lo) phase, has been proposed to describe the physical state of the lipids in lipid rafts. The lo phase is explained as an intermediate between the gel and fluid state, in which the acyl chains are still stretched (i.e., ordered) but lack a hexagonal lateral arrangement of the molecules and show fast diffusion (i.e., liquid) (Ipsen et al., 1987). For pure lipids, both these properties change simultaneously upon melting from the gel (i.e., solid ordered, so) to the fluid (liquid disordered, ld) phase, but both properties can be decoupled in the presence of Cho. It has been predicted theoretically that a lo phase can coexist in equilibrium with a ld one (Ipsen et al., 1987, 1989; Mouritsen and Jorgensen, 1994; Nielsen et al., 2000). At high Cho content of 30 mol % and more, the gel phase is typically abolished in favor of a lo phase, which is then transformed into a ld phase with increasing temperature. This applies to 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC)-Cho (Ipsen et al., 1987, 1989; Vist and Davis, 1990; Sankaram and Thompson, 1991), 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC)-Cho (Thewalt and Bloom, 1992), and 1-palmitoyl-2-petroselinoyl-sn-glycero-3-phosphocholine (PPetPC)-Cho (Miao et al., 2002; Nielsen et al., 2000), and a similar

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behavior has also been found for palmitoyl SM-Cho (Estep et al., 1979). It should be noted that the issue of lo-ld coexistence is not settled yet, and there are alternative models. To a certain extent, the problem could be resolved taking into account that the separation of lo and ld regions is limited to domains of finite, probably variable size. Depending on domain size, the system will be best described in terms of a continuous phase with intermediate properties, the formation of molecular complexes (Radhakrishnan et al., 2000; Anderson and McConnell, 2001), or two-phase coexistence.

DRMs are often tentatively identified with functional rafts, but many key issues are unresolved so far. London and Brown (2000) have emphasized that one cannot exclude that a "homogeneous intermediate state might exhibit partial detergent insolubility, and allow differential solubilization of different lipid components. This could lead to a serious misinterpretation if mistaken for the behavior of a state in which coexisting lo and L_α state domains exist." Others have brought up similar issues (Sot et al., 2002).

Experimental data presented here provide strong evidence that Triton can indeed induce or promote domain formation. That does not mean that rafts do not exist, but it means that a number of common assumptions are likely to be wrong. We will address the issue by investigating the phase behavior in an equimolar mixture of the unsaturated POPC, egg SM, and Cho in the absence and presence of TX in excess water. Without Triton, the system is assumed to form a lo and/or ld phase as discussed for similar systems (Ahmed et al., 1997; London and Brown, 2000; Dietrich et al., 2001a) and apparently all Cho-rich lipid systems studied so far (see above). A micellar phase can be formed in the presence of Triton.

The classical method to detect lipid melting is differential scanning calorimetry (DSC) (Chapman and Urbina, 1974; Blume, 1988). Anderson and McConnell (2001) have described the formation of condensed complexes in mixtures of saturated phosphatidylcholines and cholesterol in great detail using DSC. Micelle formation and dissolution could also be detected by DSC (Kresheck, 2000, 2001; Majhi and Blume, 2001). It has, however, been noted that this technique was not sensitive enough to detect the lo-ld transition in raft mixtures (Brown and London, 1997). Gandhavadi et al. (2002) reported that the lo-ld transition in DPPC-Cho could be detected by DSC, but no DSC peak could be observed in an equimolar 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC)-SM-Cho mixture. It will be shown here that state-of-the-art DSC and a new technique, pressure perturbation calorimetry (PPC) (Heerklotz and Seelig, 2002), are able to detect thermotropic changes in membrane order (such as lo→ld) as well as micellar phase (mic) to→ld transitions in the POPC-SM-Cho system.

A very potent method to study membrane solubilization upon addition of detergents is isothermal titration calorimetry (ITC) (for a review see Heerklotz and Seelig, 2000). It applies also to the micellization of the detergent-soluble fraction of the PC-SM-Cho mixture. Additionally, it reveals the Triton-driven transition within the exclusively lamellar range, which is also observed by DSC and PPC and could be assigned to domain formation.

The assignment of the different phase structures to the ranges established by microcalorimetry was aided by solid-state ^{31}P-NMR which distinguishes between lamellar and micellar structures (Seelig, 1978).

MATERIALS AND METHODS

Substances

POPC and egg SM were purchased from Avanti Polar Lipids (Alabaster, AL). Egg SM contains almost exclusively saturated, mainly palmitoyl chains. Cho and TX were from Fluka (Buchs, Switzerland). All substances were of the highest available purity and used without further purification.

Equimolar lipid mixtures were prepared by stepwise addition of the lipids from stock solutions in chloroform/methanol. After each addition of a component, the sample was dried by a gentle stream of nitrogen followed by application of vacuum overnight. This allows one to check the composition by weighing the dry material before and after each addition and to make corrections if required. Redissolution of previously added material in stock solutions added later guarantees homogeneous mixing. Finally, the dry lipid mixture was suspended in 100 mM NaCl, 10 mM Tris buffer at pH 7.4 by gentle vortexing. Large unilamellar vesicles were produced by three freeze-thaw cycles followed by 19 passages through two stacked Nuclepore polycarbonate membranes of 100-nm pore size in a mini-extruder (MacDonald et al., 1991). The sample was kept above 40°C during the extrusion procedure.

DSC

The measurements were performed on a VP DSC calorimeter from MicroCal, Northampton, MA (Plotnikov et al., 1997). The sample cell of 0.5 ml was filled with a vesicle suspension containing an equimolar mixture of PC, SM, and Cho with a total lipid concentration, [PC] + [SM] + [Cho], of at least 30 mM, typically 60 mM. Triton, if present, was added from a stock solution in matching buffer before freeze-thawing and extrusion.

Scan rates of 10 or 60 K/h were used, and the instrument was in the high-gain mode. Buffer/buffer baselines were subtracted from the curves.

PPC

The PPC measurement can be performed in the DSC instrument without refilling the cell and has been described in detail elsewhere (Lin et al., 2002; Heerklotz and Seelig, 2002). Briefly, the PPC accessory applies a pressure increase or release of at most 5 bar to the sample, which is kept at constant temperature. The compensation heat that is required to avoid a temperature decrease (pressure reduction) or increase (pressure increase) of the sample is just the reverse of the thermal response of the sample, dQ/dp at constant temperature. The relationship,

$$\left.\frac{dQ}{dp}\right|_T = T\left.\frac{dV}{dT}\right|_p, \qquad (1)$$

allows one to determine the isobaric thermal volume expansion of the lipid taking into account blank data for buffer (sample cell) versus water (reference cell), buffer versus buffer, and water versus water. A large number of such pressure jumps is performed automatically at different

temperatures yielding the expansivity as a function of temperature. The data obtained from pressure increase and decrease at the same temperature were averaged. Thermotropic phase transitions lead to a peak of the PPC curve, and the area under the peak gives the volume change accompanying the transition.

ITC

ITC was performed on a VP ITC calorimeter from MicroCal (Chellani, 1999). The solubilization protocol used here was discussed elsewhere (Heerklotz et al., 1995; Heerklotz and Seelig, 2000). Briefly, the 1.4-ml cell is filled with a mixed vesicle solution of 10 mM POPC (with or without additional SM or Cho). The syringe is loaded with 0.3 ml of a micellar solution of Triton (typically, 100 mM), and a series of injections are made by a computer-controlled step motor into the cell. To resolve both low- and high-concentration effects with one or a few runs, the injected aliquots were gradually increased from ~2 μl to 25 μl. After each injection, the heat power of reaction was recorded for 40 min to ensure reequilibration of the system. Each heat peak was integrated and normalized with respect to the mole number of Triton injected.

NMR

The phase state of the systems was determined using solid-state ^{31}P-NMR with a Bruker DRX 400 (9.4 Tesla) at 161.98 MHz. A pulse-and-acquire program with proton decoupling was used with a 90° pulse of 5 μs and a repetition time of 4 s to avoid saturation. The samples contained multilamellar vesicles of, typically, ~20–40 mg of total lipid hydrated with 20–80 mg of buffer. Three hundred to four hundred scans were recorded, and a line broadening of 150 Hz was applied.

RESULTS

NMR studies of equimolar PC-SM-Cho mixtures upon addition of Triton

Fig. 1 *A* displays ^{31}P spectra of equimolar mixtures of POPC, SM, and Cho with increasing amounts of Triton (from *left* to *right*) and at increasing temperature (from *bottom* to *top*). The Triton content will be given in terms of the mole ratio of Triton per POPC ($R_{TX/PC}$), which is just three times the value of moles of TX per mole of lipid (PC + SM + Cho). The array in Fig. 1 *A* is symbolically represented by Fig. 1 *B* where additional spectra are included to increase the resolution.

Two characteristic types of spectra are observed. All spectra above the solid line (and represented by ■) exhibit the typical powder pattern of a lamellar phase in multilamellar vesicles. There are some signs of orientation phenomena, especially at high temperature (e.g., 5 TX/PC at 90°C), but this observation is not relevant for the issue to be discussed here.

The spectra at temperatures below the solid line (or Triton contents above it) show a coexistence of the lamellar pattern and a signal that can be assigned to lipids in micelles (▽, ◇; see below). The latter corresponds to an effectively isotropic orientation of the molecules with respect to the external field as a consequence of fast angular reorientation. At least some lamellar structures remain throughout the whole temperature and composition range investigated here, which is in accord with the fact that the system forms so-called DRMs.

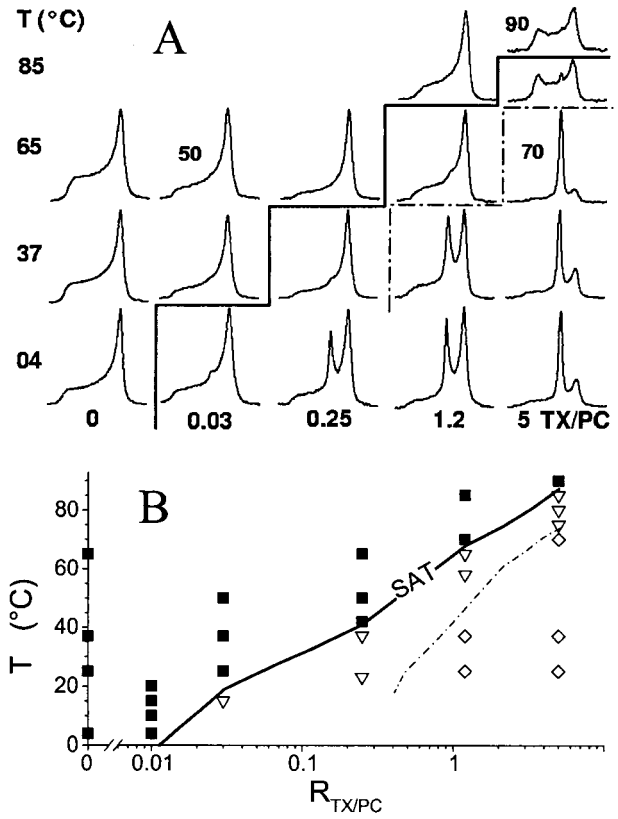

FIGURE 1 ^{31}P solid-state NMR spectra (*A*) and symbols representing these and other spectra (*B*) of an equimolar mixture of PC, SM, and Cho as a function of Triton content $R_{TX/PC}$ (*horizontal*) and temperature T (*vertical*). Spectra recorded at temperatures differing from the number at the left margin of *A* are individually labeled (50°C, 70°C, and 90°C, respectively). A solid line shown both in *A* and *B* (denoted with *SAT* in *B*) separates exclusively lamellar powder patterns (■ in *B*) from coexisting lamellar and micellar signals (▽ or ◇ in *B*). ▽, spectra exhibiting a substantially smaller relative intensity of the micellar signal compared with the same sample at the next lower temperature measured; i.e., they imply a reconstitution of membranes at the expense of micelles with increasing temperature. ◇, spectra with virtually unchanged or increasing micellar fraction than at lower temperature. The lower-temperature boundary of the range of thermal reconstitution is illustrated by a dash-dot line.

At first glance, it becomes obvious that the critical amount of Triton required for the onset of solubilization, i.e., the membrane saturation (SAT) boundary, depends strongly on temperature. With increasing temperature, increasing amounts of Triton are needed to initiate mixed micelle formation. A closer inspection of the data reveals that the lamellar/micellar coexistence range can be further divided into two regions according to characteristic changes in the relationship between lamellar and micellar signals. Let us, for example, consider the column of spectra at 5 TX/PC in Fig. 1 *A*. At 90°C, a lamellar pattern is observed exclusively so that the spectrum is represented by ■ in Fig.

1 *B*. Upon cooling to 85°C, solubilization is detected to start (i.e., the system crosses the SAT boundary) by the appearance of a small micellar signal on top of the lamellar pattern. Such a spectrum is represented by an open symbol and that ▽ is used rather than ◇ means that solubilization continues below 85°C. This is deduced from the fact that a larger micellar signal is obtained at the next lower temperature, 80°C (spectrum not shown). This growth of the micellar fraction proceeds also upon cooling to 75°C (▽) but comes to a halt at 70°C (◇) where even a somewhat larger micellar signal is observed than at 37°C. The lower limit of the partial membrane solubilization is indicated by the dash-dot boundary. The spectrum at 25°C shows equal or more micelles than at 4°C, again denoted by ◇. The spectrum at 4°C could not be included in Fig. 1 *B* because no reference spectrum at lower temperature was available.

It must be emphasized that the lipid concentration in the NMR sample is large enough to ensure an almost complete incorporation of Triton into the membrane (or micelles), and Triton monomers in aqueous solution can be neglected. Therefore, the total $R_{TX/PC}$ is a good approximation for the local Triton content in membranes or membranes and micelles (mean value), often referred to as R_e.

ITC

ITC is a standard method to detect membrane solubilization by detergents. To illustrate the typical behavior for homogeneous fluid membranes, the ITC curve of pure POPC (10 mM) titrated with Triton at 37°C is shown as a thin line in Fig. 2 (*center panel*). It exhibits the typical three-stage behavior (Heerklotz et al., 1995; Heerklotz and Seelig, 2000). In the first stage, injected micelles dissolve and the Triton incorporates into the membrane. This transfer is endothermic by ~2 kcal/mol. The onset of solubilization is indicated as a sudden drop of the titration heat to exothermic values at $R_{TX/PC}^{SAT} = 0.42$ and leads into the range of coexisting micelles and membranes. Solubilization is completed beyond another breakpoint at 2 TX/PC, and additional injections give rise to small endothermic heats (presented titration ends right after break point).

A more complex pattern is obtained for the raft mixture. The incorporation of Triton into the mixed membrane is much more endothermic, but the heat of transfer decreases steeply in the course of the titration until a sharp minimum is reached at 0.12 TX/PC (at 37°C) followed by an increase of the heat of titration until ~0.16 TX/PC. This drop of the ITC curve resembles to some extent the solubilization effect in pure POPC and without additional information could be misinterpreted as a sign of membrane solubilization. However, the NMR spectra recorded at 37°C (cf. Fig. 1) show that no micelles are formed below ~0.2 TX/PC. It must therefore be concluded that the transition seen by ITC at ~0.12 TX/PC occurs between different lamellar structures that give rise to very similar ^{31}P-NMR spectra. This could indicate either a gradual change in membrane order in general or in the proportion of coexisting lo and ld domains.

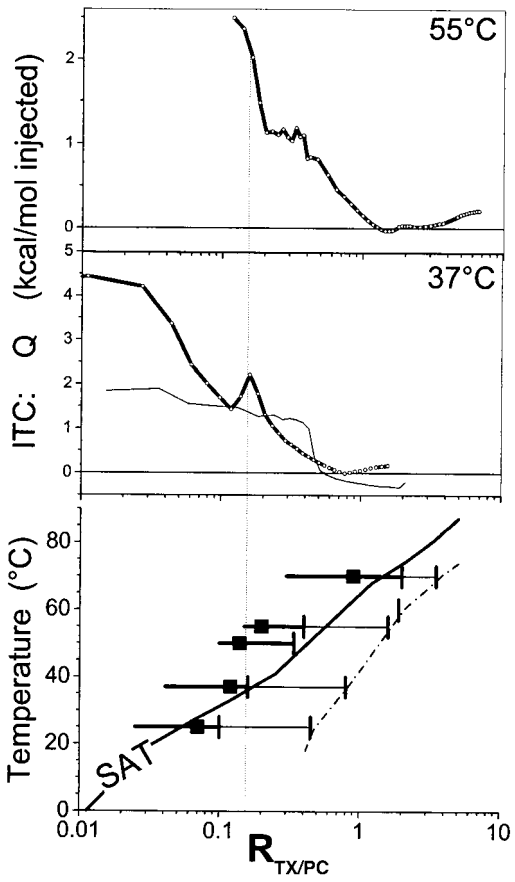

FIGURE 2 Results of ITC solubilization experiments at 55°C (*upper panel*) and 37°C (*middle panel*). The bold lines and circles indicate the heats per mole of micellar Triton injected into an equimolar mixture of POPC-SM-Cho (30 mM). The thin solid line shows the ITC curve for Triton injection into pure POPC vesicles (10 mM). The bottom panel shows a symbolic representation of these and other ITC runs with horizontal lines (*bold* with *square* at the peak, and *thin* with *bars* at the ends) representing transition ranges suggested by discontinuities of the ITC curves. The phase boundaries established by NMR (*solid* and *dash-dot lines*; see Fig. 1 for details) are given for comparison.

The solubilization predicted by NMR between ~0.2 and 1.2 TX/PC is also observed by ITC as another drop of the ITC curve. At 55°C (cf. Fig. 2, *top panel*), the two transitions are observed at higher Triton contents, respectively, and the heat of titration does not really increase after the first, lamellar-to-lamellar transition but, after a break point, decreases again because of membrane solubilization. The bottom panel of Fig. 2 compares the transition ranges observed in ITC experiments at various temperatures with the phase boundaries derived from NMR (cf. Fig. 1 *B*). The lamellar transitions are depicted by bold lines with a square at the maximum position and the partial membrane solubilization by thin lines. An almost perfect agreement of the NMR and ITC data is observed above room temperature,

where Triton is almost fully incorporated into the membranes and/or micelles.

To summarize the major result from ITC, this method reveals also the range of partial membrane solubilization in a DRM-forming mixture of POPC-SM-Cho. However, it gives also good evidence for another transition driven by Triton within the exclusively lamellar range.

DSC/PPC

The strong temperature dependence of the phase boundaries shown in Figs. 1 and 2 suggests that these transitions could also be driven by temperature changes and, thus, studied by DSC and PPC.

Whereas ITC keeps the temperature constant and changes the Triton content, both DSC and PPC monitor effects with varying temperature at constant sample composition. Hence, the bottom panel of Fig. 3, which summarizes the DSC and PPC results, is equivalent to those of Figs. 1 and 2 but with ordinate and abscissa exchanged.

The other panels of Fig. 3 show chosen PPC and DSC curves of an equimolar mixture of POPC-SM-Cho with four different Triton contents as specified in the plot. The transitions are fully reversible as indicated by the fact that repetition of DSC and PPC runs yielded identical results. In the absence of Triton (*fourth panel*), both techniques reveal a transition between <0°C and $\sim$60°C with a maximum at $\sim$20°C. It is depicted in the bottom panel of Fig. 3 by a bold bar with a square at the peak position. It should be noted that the interpretation of such broad peaks is not straightforward. First, they might not represent only first-order phase transitions involving a coexistence of rather well separated phases but also the formation/dissolution of small domains or even a gradual transformation of a homogeneous membrane phase. Second, the determination of the upper and lower boundary of such broad phase transitions from a DSC curve has a rather limited precision (cf. Blume, 1988). Third, an integration of DSC and PPC peaks to determine ΔH and ΔV is hindered by the problem of computing a realistic baseline. For the conclusions drawn in the present study, these problems are of minor importance. Although no strict decision can be made on the basis of the available data, it will be argued below that the domain-formation model seems to be more suitable to discuss the behavior of the membrane, at least in the presence of Triton, than the idea of a continuous transformation of a homogeneous membrane.

The thermotropic behavior of the lipid mixture in the presence of 0.25 TX/PC is shown in the third panel of Fig. 3. Two peaks are observed by DSC as well as PPC. The temperature range of the first peak, $<4-40$°C, corresponds quite well to the range where NMR reports a thermotropic transition from a micelle-lamellae coexistence to an exclusively lamellar structure (cf. *third column* of Fig. 1 *A*). The reconstitution of the micellar fraction to membranes may be

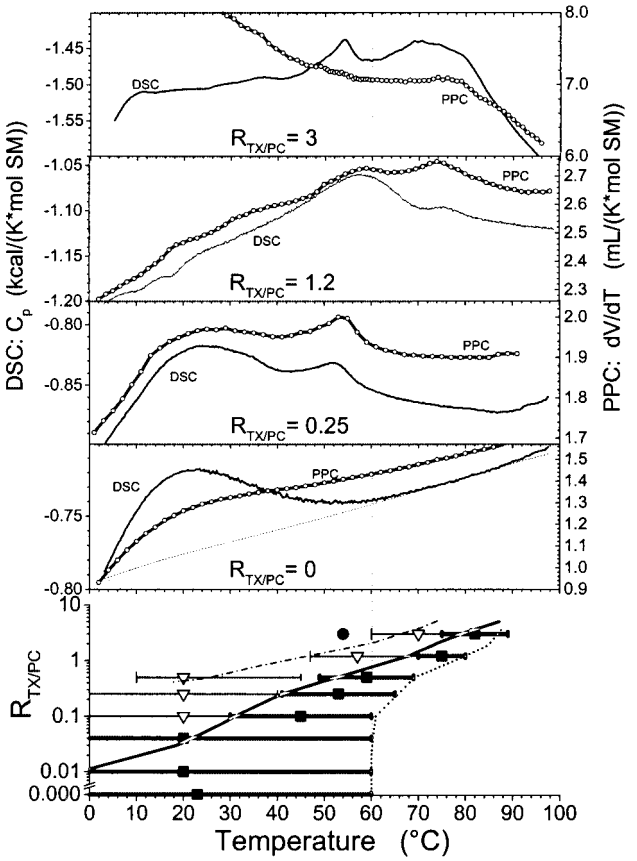

FIGURE 3 The isobaric heat capacity, C_P (i.e., *DSC curve*, *solid lines*, *left axis*) and thermal change in partial molar volume of the lipid/detergent system, dV/dT (i.e., the *PPC curve*, *bold line* with ○, *right axis*) versus temperature for an equimolar mixture of POPC-SM-Cho containing variable amounts of Triton (*top* through *fourth panel*) as specified in terms of the mole ratio $R_{TX/PC}$ in the plot. The bottom panel summarizes these and other experiments showing peak positions (■, ▽, and ●) and transition ranges (*bold* and *thin bars*). A crude approximation for the upper boundary of the lamellar transition (■) is added as a dotted line to the solid and dash-dot phase boundaries established by NMR (see Fig. 1).

paralleled by a partial melting of lo domains to ld. This would, at least, explain the similarity of the size and shape of the DSC/PPC peaks below 40°C in the absence and presence of 0.25 TX/PC.

A major change is, however, caused by addition of 0.25 TX/PC in the behavior above 40°C. DSC and PPC show a sharp peak (Fig. 3, *third row*), whereas NMR indicates that no micelles are present and structural changes do not vary the spectra. Assuming that this peak represents a lo to ld transition, one must conclude that the presence of Triton stabilizes at least part of the lo phase so that it melts to ld at a rather characteristic, enhanced temperature of $\sim$54°C. The fact that the PPC/DSC runs at 0.04 TX/PC fail to resolve two separate peaks (curve not shown) must be explained by incomplete membrane partitioning at low temperature.

At a rather high Triton content of 1.2 TX/PC there is no clear sign of a transition enthalpy or volume at <40°C.

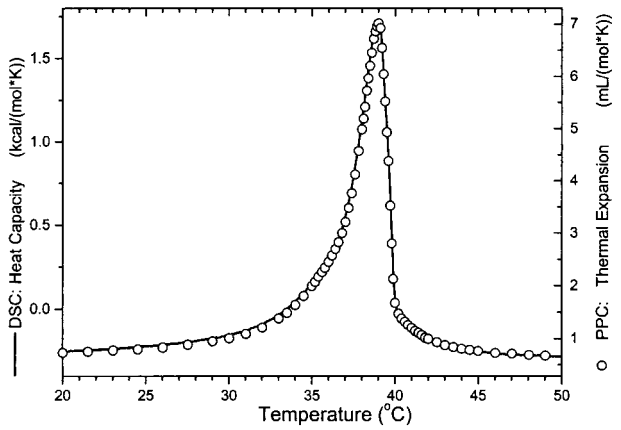

FIGURE 4 DSC (*solid line, left axis*) and PPC (○, *right axis*) curves of pure egg SM vesicles (extruded). The methods reveal the gel (so)→ld phase transition at 39°C. The areas underneath the peaks yield the accompanying enthalpy (DSC) and volume (PPC) changes (see text).

There, the micellar NMR signal is also not reduced with increasing temperature. The onset and completion of thermal membrane reconstitution are at ~40°C and 70°C as observed consistently by NMR, DSC, and PPC. A transition between two lamellar states occurs between ~70°C and 80°C.

At 3 TX/PC, comparison of the DSC, PPC, and NMR data implies that membrane reconstitution and lamellar transition give rise to overlapping peaks between ~60°C and 90°C. As an additional, new phenomenon, a DSC peak is obtained at 55°C (see ● in *bottom panel*), which is not paralleled by any volume change (i.e., PPC peak). The latter phenomenon might arise for instance from changes in the micellar geometry, but a detailed interpretation is beyond the scope of this study.

To summarize the DSC and PPC results, these techniques detected the onset and completion temperatures of thermal membrane reconstitution in almost perfect agreement with the predictions from NMR and ITC (phase boundaries in Figs. 1 *B*, 2, and 3 are identical). The presence of Triton seems to stabilize a more ordered state of the membrane as indicated by an up-shift of the transition or transformation temperature.

Melting of pure SM vesicles

The melting characteristics of pure SM vesicles were studied with DSC and PPC for the sake of comparison (Fig. 4).

The two techniques yield transition curves of virtually identical shape (cf. Grabitz et al., 2002) with a maximum at 39.0°C and a half-width of 2.2 K. Integration of the DSC curve yields an enthalpy of melting, i.e., for the gel- to liquid-crystalline transition, of $\Delta H = 7.3$ kcal/mol. Integration of the PPC peak allows one to quantify the accompanying volume change to $\Delta V = 21$ ml/mol. The latter value corresponds to a relative volume change of ~3.0 vol % assuming a partial molar volume of 1 ml/g. The Clausius Clapeyron equation predicts the pressure dependence of the transition to $\Delta T_m/\Delta p = T_m \times \Delta V/\Delta H = 22$ K/kbar (using 1 J/m^3 = 10^{-8} kbar).

DISCUSSION

Different structural preferences of TX and SM promote domain formation

The major issue of this study is whether the application of Triton allows the isolation of lo domains in their original state from raft mixtures or whether the detergent interferes with domain formation. The most important observation for this problem is the finding that either a phase transition between two lamellar states or a certain transformation of the lamellar state is driven by cooling as well as by addition of Triton. It is a general fact that cooling promotes order because of a reduced effect of entropy on the equilibrium state. Hence, it is strongly counterintuitive at first glance that a strong detergent that is well known to disorder membranes would have a similar effect as cooling. Whereas it reduces the melting temperature of pure PC membranes (Goni et al., 1986), it increases the temperature of a transition or transformation in the PC-SM-Cho membrane. There seems, indeed, to be no convincing explanation for this phenomenon if the membrane is considered to be one homogeneous phase where Triton addition and ordering must occur simultaneously. If the ITC, DSC, and PPC peaks found within the exclusively lamellar phase could, however, be described approximately in terms of a phase transition involving at least partial phase separation into different, e.g., ld and lo domains, the apparent paradox would be easily resolved. Then, Triton would disorder one phase whereas the other, Triton-free phase is ordered. This idea is supported by a more detailed inspection of the forces governing mixing versus de-mixing in a membrane.

Fig. 5 shows a tentative phase diagram of the equimolar mixture of POPC-egg SM-Cho and Triton as a function of temperature based on the bottom panels of Figs. 1–3 and the assumption of a lo-ld transition involving coexisting domains.

The problem of Triton-induced domain formation and membrane solubilization will be discussed in the following by a detailed inspection of the systems represented by the points labeled in Fig. 5, *a–f*.

Point *a* in Fig. 5 corresponds to the lipid mixture without Triton at 37°C. There is assumed to be lo + ld coexistence, and part of the SM resides, along with Cho, in ordered domains. The ordered chains have a larger projected length so that the bilayer thickness of the lo domains is somewhat larger (Gandhavadi et al., 2002). This tightly packed state improves the interactions between the molecules and is enthalpically favored. In contrast, entropy favors mixing

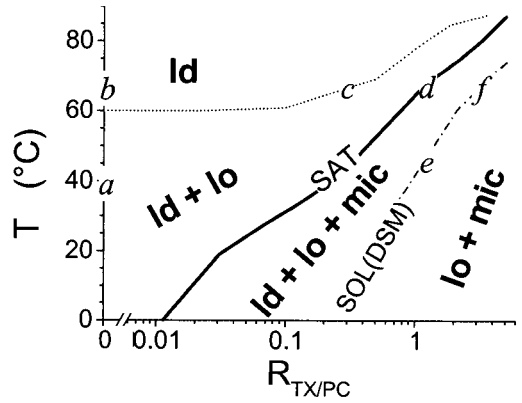

FIGURE 5 Tentative phase diagram for an equimolar mixture of POPC-SM-Cho depending on Triton content (mole ratio $R_{TX/PC}$) and temperature (T) constructed on the basis of the bottom panels of Figs. 1–3. The ITC, DSC, and PPC peaks within the exclusively lamellar range are assumed to represent a transition involving coexisting lo and ld domains. This assumption and the points labeled *a–f* are discussed in the text.

and causes part of the SM and Cho to remain in the PC-rich, ld phase despite the enthalpic penalty of disordering.

The contribution of the entropy to the Gibbs free energy and thus its effect on the equilibrium state of the system is proportional to temperature. Hence, more and more SM and Cho are transferred into the mixed ld phase upon increasing temperature. At 65°C (point *b*), the system is fully controlled by the entropy term and the lo domains have vanished.

Now, let us imagine that Triton is added stepwise to the membrane, thus moving from *b* toward *c*. The detergent fluidizes the membrane, and the order of the chains and their projected length are reduced. This is in conflict with the preferences of (in particular) SM and Cho and increases the enthalpic penalty of SM and Cho localization within the disordered phase, an effect that is quantitatively reflected by the enthalpy of incorporation of Triton into PC-SM-Cho (~4 kcal/mol at 37°C; cf. Fig. 2) compared with POPC (~2 kcal/mol). The entropy term remains essentially unchanged so that also the partial Gibbs free energy of Triton uptake into SM is increased compared with PC as found in partitioning studies (Nyholm and Slotte, 2001).

At a Triton content of ~0.3 TX/PC (point *c*), the enthalpic penalty of TX-SM mixing becomes large enough to compete with the entropy term, and the first SM and Cho molecules form Triton-depleted lo domains. The formation and/or growth of lo domains proceeds up to point *d* when the SAT boundary is reached and the composition of the lo domains may change because of different effects of Triton on SM and Cho.

Beyond this boundary, additional Triton starts to form mixed micelles and is no longer incorporated into membranes (see below). It is noteworthy that this process can also be understood in terms of the phase separation concept explained above. Accordingly, the most unfavorable SM-TX and/or Cho-TX interactions cause a first phase separation into SM/Cho and PC/TX-rich phases. The less unfavorable PC-TX interactions cause a further separation of the PC/TX-phase into a PC-rich (lamellar) and a TX-rich (micellar) phase.

So far, it must be emphasized that the addition of Triton may create ordered domains even in an initially homogeneous, fluid PC-SM-Cho membrane. Preexisting lo domains (e.g., in our system at 37°C) may grow in size or number upon application of Triton unless another, biological domain promoter is similarly active in the natural system. DRMs isolated from biological membranes are expected to overestimate ordered domains or might even be obtained from raft-free membranes.

Membrane solubilization

Starting at the SAT boundary, solubilization of lipids into mixed micelles occurs upon further addition of Triton as well as upon decreasing temperature.

If the system is cooled down from point *d* in Fig. 5 (see also Fig. 1 *A*, *fourth column*), solubilization comes to a halt at ~45°C (Fig. 5 *e*). The notation SOL(DSM) for the phase boundary is based on the conclusion that the solubilization of the detergent-soluble membrane fraction (DSM) is completed at this temperature, leaving only DRMs behind. DRMs are usually identified with lo domains.

If the system is, instead, kept at 65°C and more Triton is added, the SOL(DSM) boundary is reached at ~3 TX/PC (Fig. 5 *f*). Note that cooling drives both a ld→mic and a ld→lo transition so that the DRM fraction in Fig. 5 *e* (approximately represented by Fig. 1 *A*, 1.2 TX/PC at 37°C) is larger than in *f* (approximately represented by 5 TX/PC at 70°C). This observation is in accord with the fact that DRM isolation from biomembranes has a better yield at lower temperature.

At least at 37°C (Fig. 2), it must be stated that less Triton is required per PC for the onset and completion of solubilization of the DSM fraction compared with pure POPC. This is in line with the finding that a gradual reduction of turbidity accompanying solubilization occurs already at lower Triton concentration in the presence of SM and Cho (Sot et al., 2002).

It should be mentioned that the resistance of lo domains to solubilization is not absolute. The composition of the DRM fraction must also be considered to be dependent on temperature and detergent concentration. Additional Triton in the lo + mic range will lead to DRM solubilization until, eventually, another phase boundary, SOL(DRM), to an exclusively micellar range is encountered at concentrations above the range investigated here. This effect is also reflected by the variation of the NMR spectra from, e.g., 1.2 to 5 TX/PC at 4°C (Fig. 1 *A*). Furthermore, the growth of the micellar signal with temperature at 5 TX/PC within the lo + mic range from 37°C to 70°C (Fig. 1 *A*) can be taken

as a clue that the inclination of the lo + mic/mic boundary is toward smaller $R_{TX/PC}$ with increasing temperature (opposite to SOL(DSM)) so that heating may drive the system somewhat toward the exclusively micellar range. In other words, whereas DSM solubilization proceeds upon cooling, DRM solubilization is expected to proceed upon heating.

Comparison with DRM isolation

The yield and selectivity of DRM isolation from biomembranes is therefore supposed to be maximum at low temperature and Triton contents slightly above the SOL(DSM) boundary. Unfortunately, a quantitative comparison between the partial solubilization of cell and model membranes is by no means straightforward. It requires estimating the effective lipid concentration of the cell suspension and the membrane-bound amount of Triton. Yu et al. (1973) solubilized erythrocyte ghosts in a suspension of six times the net volume of the cells. This corresponds to a lipid concentration on the order of 1 mM taking into account a volume of 90 μm^3 per erythrocyte, a surface area of 140 μm^2 (Pantaler et al., 2000), and two accessible layers that are up to 67% covered by lipid of 65-$Å^2$ lateral area requirement.

Yu et al. (1973) observed the solubilization of the soluble membrane fraction to start between 0.1 and 0.7 mM TX and to be completed between 1.4 and 7 mM TX. Partitioning studies of TX between buffer and the lipid mixture studied here (to be published) suggest that at 4°C and 1 mM lipid, only on the order of 5% of the Triton will be membrane bound and thus available for membrane solubilization. If one applies this value as a crude approximation to the erythrocyte data, DSM solubilization is supposed to start at ~0.005–0.04 TX/lipid and to be completed at ~0.05–0.4 TX/lipid.

This is in good agreement with the corresponding phase boundaries in our model system, which are ~0.003 and 0.2 TX/lipid (1/3 of $R_{TX/PC}$, respectively). Despite the uncertainties of this estimate, we can conclude that our study is performed in the biologically relevant concentration range and that our model system shows a similar solubilization behavior as erythrocytes.

Implications for lipid rafts

Only a minor part of the lipid mixture studied here is in an ordered state at 37°C, but small concentrations of a membrane-disordering additive (in our case some mole percent of Triton) can greatly enhance domain formation by a rather unspecific mechanism. This implies that rafts in biological membranes containing a large variety of molecules should not be considered as steady particles the number and size of which depends only on SM and Cho content and temperature. Instead, they might form or disappear, grow or shrink, and cluster or break up triggered by small amounts of other compounds or other effects such as structural changes of proteins interfering with lipid packing.

Recent studies have indeed challenged the idea of large, long-term stable rafts and suggested that rafts are originally small and contain only a small subset of raft-associated proteins so that raft clustering is required to enable typical functions (Simons and Toomre, 2000).

The fact that the addition of Triton promotes domain formation and may even create domains in a homogeneous fluid lipid mixture argues strongly against an identification of DRMs with functional rafts.

Another lesson from the phase diagram is that the temperature dependence of domain formation changes substantially with increasing Triton content. That means also that there could be rafts in a membrane at 37°C, although it does not yield DRMs at this temperature.

CONCLUSIONS

DSC, PPC, and ITC can be used to detect domain formation as well as partial solubilization in mixed vesicles of PC, SM, Cho, and Triton.

The behavior of an equimolar mixture of POPC-SM-Cho can be explained in terms of a typical sequence of the phase ranges ld, ld + lo, ld + lo + mic, and lo + mic with increasing Triton content or decreasing temperature (in the presence of Triton). That means that addition of Triton promotes the formation of lo domains.

Hence, DRMs should not be expected to resemble functional rafts regarding their abundance, size, composition, or even existence.

Functional rafts should not be considered as rather steady phenomena but may appear and vanish or change their properties markedly upon rather subtle changes in membrane packing.

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