Chemistry and Physics of Lipids 167–168 (2013) 57–61

Contents lists available at SciVerse ScienceDirect

Chemistry and Physics of Lipids

journal homepage: www.elsevier.com/locate/chemphyslip

The onset of Triton X-100 solubilization of sphingomyelin/ceramide bilayers: effects of temperature and composition

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ARTICLE INFO

Article history:
Received 4 January 2013
Accepted 6 February 2013
Available online 20 February 2013

Keywords:
Membrane solubilization
Detergents
Sphingomyelin
Ceramide
Triton
Isothermal calorimetry

ABSTRACT

The early stages of Triton X-100 solubilization of bilayers consisting of sphingomyelin/ceramide (SM/Cer) mixtures have been studied using a combination of calorimetric and spectroscopic techniques. Compositions based on sphingomyelin, containing up to 30 mol% Cer, at 4, 20 and 50 °C have been examined. The presence of Cer does not modify the affinity (in terms of ΔG of binding per mol total lipid) of the SM-based bilayers for Triton X-100, although it does increase the amount of detergent required for the onset of solubilization. At 50 °C more detergent was required to solubilize the SM/Cer bilayers than at 20 °C. The data can be rationalized in terms of lipid and detergent geometries and interactions (Lichtenberg et al., 2013).

1. Introduction

Detergents are useful tools in the study of biomembranes, yet their use remains largely empirical, in spite of important advances in the kinetics of solubilization and the mechanism of action (Helenius and Simons, 1975; Heerklotz, 2008; Lichtenberg et al., 2013). From the early studies it became clear that not all lipids or proteins were solubilized under the same conditions, and that a non-solubilized fraction often remained after detergent treatment (Yu et al., 1973; Gurtubay et al., 1980). The detergent-resistant membrane fraction has been often mistaken for the microdomains called "rafts" (Heerklotz, 2002; Lichtenberg et al., 2005). The origin of detergent resistance, particularly for the case of Triton X-100 and pure lipid bilayers has been experimentally examined in the last decade. Studies from this and other laboratories have shown that sphingomyelin (SM) bilayers require less detergent for solubilization than those based on phosphatidylcholine (PC) (Patra et al., 1999; Ollila and Slotte, 2002; Ahyayauch et al., 2009), that bilayers in the liquid-ordered (l_o) state are more resistant to solubilization than liquid-disordered (L_α) ones (Sáez-Cirión et al., 2000), and that bilayers consisting of a single phospholipid in the gel state (L_β) are solubilized more easily than those in the L_α phase (Schnitzer et al., 2003; Ahyayauch et al., 2012). However the binary mixtures containing saturated PC or SM and ceramide give rise to highly resistant bilayers in the gel phase (Sot et al., 2006; Busto et al., 2010).

The different solubilization of gel and fluid phases has been the object of detailed experimentation. Arnulphi et al. (2007) measured the binding of Triton X-100, at sub-solubilizing concentrations, to SM or dipalmitoyl PC (DPPC) in the gel and fluid phases, using isothermal calorimetry. ΔG of binding was virtually the same for bilayers in the gel and in the fluid phase, these data arguing against a different affinity of those two phases for Triton X-100 as the origin of the observed easier solubilization of the L_β phase (Arnulphi et al., 2007). If it was not due to a higher affinity of the gel phases for Triton X-100, the easier solubilization of L_β as compared to L_α bilayers (Schnitzer et al., 2003; Patra et al., 1998) could perhaps be explained by events taking place downstream of detergent binding, such as bilayer saturation with detergent and subsequent onset of solubilization. Ahyayauch et al. (2012) showed that this was indeed the case, i.e. that bilayers in the gel phase became detergent-saturated at lower Triton X-100 concentrations than those in the L_α phase, so that this could explain at least partially the easier solubilization of the L_β phase.

The present study is aimed at studying the solubilization of bilayers composed of SM and ceramide (Cer) mixtures. These mixtures are relevant in relation to the physiological events of SM degradation to Cer in the sphingolipid signalling pathway (Hannun et al., 1986; Kolesnick, 1987; Kolesnick et al., 2000). As discussed above, SM/Cer mixtures are much more difficult to solubilize than pure SM (Sot et al., 2006). We have measured Triton X-100

Abbreviations: Cer, ceramide; DPPC, dipalmitoylphosphatidylcholine; ITC, isothermal titration calorimetry; LUV, large unilamellar vesicles; MC540, merocyanine; MLV, multilamellar vesicles; PC, phosphatidylcholine; SM, sphingomyelin.

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http://dx.doi.org/10.1016/j.chemphyslip.2013.02.003

binding to vesicles composed of SM/Cer at sub-solubilizing detergent concentrations using isothermal calorimetry. The onset of solubilization of these bilayers has also been measured by turbidity changes, or through changes in merocyanin 540 absorption spectra (Ahyayauch et al., 2012; Kaschny and Goñi, 1992). Our results show that the presence of up to 30 mol% Cer does not modify the affinity of SM bilayers for Triton X-100, although it does increase the amount of detergent required to cause bilayer solubilization, thus onset of solubilization.

2. Materials and methods

2.1. Materials

Egg sphingomyelin (SM) and ceramide (Cer) were supplied by Avanti Polar Lipids (Alabaster, AL). Triton X-100 (batch no. 125H0569) and merocyanine 540 (MC540) were purchased from Sigma (St. Louis, MO). All other reagents were of analytical grade.

2.2. Preparation of large unilamellar vesicles (LUVs)

The lipids were dissolved in chloroform:methanol (2:1, v/v) and mixed as required, and the solvent was evaporated exhaustively. Multilamellar vesicles (MLV) were prepared by hydrating the dry lipids in buffer, with vortex shaking. Lipids were hydrated in 10 mM HEPES, 150 mM NaCl, pH 7.4. Large unilamellar vesicles were prepared by the extrusion method (10 passages) with filters 0.1 μm in pore diameter (Mayer et al., 1986). Vesicle size was measured by quasi-elastic light scattering in a Malvern Zeta-Sizer 4 spectrometer. The average diameter of the vesicles was in all cases ca. 100 nm.

2.3. Turbidity assay

Liposome dispersions were mixed with the same volumes of the appropriate Triton solutions in the same buffer. Final lipid concentration, measured as lipid phosphorus, was ranging from 0.3 mM to 2.0 mM. The mixtures were left to equilibrate for 1 h at the desired temperature, and solubilization was assessed from the changes in turbidity (Goñi and Alonso, 2000) measured as absorbance at 500 nm in a double-beam Uvikon Kontron spectrophotometer (Kontron Instruments, Milan, Italy). Temperatures were kept constant with less than 0.5 °C deviation.

2.4. Absorption spectra of merocyanine (MC540)

Due to the 'cloud point' of Triton X-100, at ≈50 °C, membrane solubilization was monitored following detergent-induced changes in the visible absorption spectrum of MC540 (Kaschny and Goñi, 1993). This dye is known to show a shift in the absorption maximum wavelength (λ_{max}) in the presence of micelles (Smith, 1990; Kaschny and Goñi, 1992). The lipid and MC540 (at a molar ratio 250:1) mixtures in organic solution were evaporated under vacuum for 2 h. The LUVs were prepared as above. The liposome dispersions containing MC540 were mixed with the same volume of the appropriate Triton solutions. Absorption spectra were recorded between 400 and 600 nm with a double-beam Uvikon Kontron spectrophotometer.

2.5. Isothermal titration calorimetry (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. Stirring at a constant 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 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 (Seelig, 1997; Heerklotz and Seelig, 2000).

3. Results

3.1. Triton X-100 partitioning into SM/Cer bilayers

Triton binding to LUVs composed of pure egg SM or egg SM/Cer mixtures were measured by ITC at 4, 20, and 50 °C. The procedure was detailed in Arnulphi et al. (2007). The experimental results were converted into plots of heat exchanged/mol lipid as a function of lipid/detergent mole ratio (Fig. S1). Dilution heat (≤5% of total heat exchange) was subtracted. The results were analysed in terms of a partition model (Seelig and Ganz, 1991) that is based on the assumption of a linear relationship between the concentration of surfactant free in solution and the concentration of membrane-bound surfactant (Heerklotz and Seelig, 2000). The partition model curves fitted the experimental values with $r^2 > 0.99$.

The calorimetric data are summarized in Table 1. The molar partition constants K are very similar, in the range 0.5–1.2 mM^{-1}, irrespective of temperature or Cer concentration in the bilayers. Consequently ΔG of partition remains in the −24/−27 kJ/mol range in all cases. Only for pure SM at 50 °C ΔG is slightly more negative (−29.5 kJ/mol) but even this difference can hardly affect the amount of detergent required for solubilization (see below). We conclude that, just as in the case of pure SM, or pure DPPC (Arnulphi et al., 2007), in SM/Cer mixtures Triton X-100 partitioning into the lipid bilayers is independent from temperature, in the 4–50 °C range. (At 30 mol% Cer and 50 °C no ITC measurements could be performed because that temperature is near the midpoint transition temperature of the SM:Cer (70:30 mol ratio) mixture, and under those conditions the ITC data cannot be fitted to a simple model.) Also as for pure SM or pure DPPC (Arnulphi et al., 2007) the observed constant ΔG values were the result of mutually compensating changes in ΔH and TΔS, the enthalpic component becoming negative at the higher temperatures, when entropy change was made smaller, while remaining positive, at those elevated temperatures (Fig. 1 and Table 1). The conclusion is that any change in SM bilayer solubilization by Triton X-100 as a result of the presence of Cer cannot be attributed to a differential partition of the surfactant, i.e. Cer does not appear to interfere with Triton X-100 partitioning into SM-based bilayers.

3.2. Solubilization of SM/Cer bilayers in the gel and fluid phases

Triton X-100 solubilization of bilayers composed of pure egg SM or of SM/Cer at 95:5 or 75:25 mol ratios was measured at 20 and 50 °C. Detergent concentrations required to cause the onset of solubilization (D_{on}) were measured at 20 °C by the turbidimetric method (see Fig. S2 for a representative experiment) while the merocyanin 540 procedure was used at 50 °C to avoid cloud-point