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THERMOTROPIC PROPERTIES OF BIPOLAR LIPIDS OF *SULFOLOBUS SOLFATARICUS* AND OF THEIR MIXTURES WITH DIPALMITOYLPHOSPHATIDYLCHOLINE

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The thermotropic properties of the bipolar lipids, glycerol dialkylglycerol tetraether (GDGT) and glycerol dialkylnonitol tetraether (GDNT), were determined at different degrees of hydration and in mixtures with dipalmitoylphosphatidylcholine (DPPC). The number of water molecules rendered unfreezable by the GDNT molecule is 10 ± 1.5 and that by the GDGT molecule 2.8 ± 0.7 or about 1.1–1.5 H_2O molecules per OH group. Binding of water molecules causes randomization of the two polar heads from the oriented form prevailing in the dry state. The hydration seems to be a cooperative process extending over a whole lipid domain. DPPC added in small amounts to GDNT interacts preferentially with the nonitol halves of the molecules separating them from the glycerol half molecules. In the cooperative interaction domain each DPPC molecule is surrounded by up to six GDNT molecules. Cooperative domains formed during the interaction of DPPC with GDGT are less pronounced. In both cases they affect the thermotropic properties of the system.

1. Introduction

The lipids of the plasma membrane of the thermoacidophilic archaebacterium, *Sulfolobus solfataricus*, previously named *Calderielle acidophila*, have two polar heads – either two glycerols or one glycerol and one nonitol linked together by two C_{40} biphytanyl chains [2–4]. The biphytanyl chains contain up to eight cyclopentane rings per lipid molecule. *S. solfataricus* can be grown between 75 and 90°C, and the number of rings per molecule increases with the growth temperature [4].

Unlike other phosphatidylcholines, the synthetic 1,2-biphytanylphosphatidylcholine does not undergo phase transition between -120 and $+120$°C [5]. It is believed that the lack of transition into the rigid (gel) state is caused by the steric effects and stems from the very expanded structure of the molecule due to the methyl groups at the branch points along the hydrocarbon chains. DSC measurements of biphytanoyl tetraether glycolipids from *Thermoplasma aeidophilla* showed [6] that they undergo phase transition at -20°C. The thermotropic properties of dry (unhydrated) glycerol dialkylnonitol tetraethers (GDNT) containing four, five and six cyclopentane residues per lipid molecule were investigated by DSC (4-GDNT, 5-GDNT and 6-GDNT). The lipids undergo two phase transitions with their temperature of melting increasing with the number of rings [8]. It is evident from the published results that the five-membered rings contribute strongly to the cohesive forces between the hydrocarbon chains, presumably due to the tendency of the rings in the neighboring chains to form stacked conformations. This stacking should be strongly affected by addition of the lipids tending to break the stacked forms. The degree of stacking may also affect the interaction between the polar groups and their hydration (water binding). In the present work we

have investigated the effect of dipalmitoylphosphatidylcholine (DPPC) on the thermotropic properties of the glycerol dialkylglycerol tetraethers (GDGT) and (GDNT), as well as their hydration. However, as nothing was known about the thermotropic properties of the bipolar lipids in an excess of water and about water binding to these lipids, an initial part of this study was devoted to answering these questions.

In this investigation we used the lipid mixtures extracted from bacteria grown at one temperature. The lipids differing in the head groups were separated. However, no separation was performed based on the number of cyclopentane rings in the hydrocarbon chains. As one can see from fig. 1, the location of the rings is fixed with respect to the polar heads. The first rings appear after the seventh carbon counting from the ether oxygen. The consecutive rings are located near the first ring closer to the polar head. Thus, full or partial stacking between rings of neighboring lipid molecules is possible even if the hydrocarbon chains are not identical. Bacteria grown at different temperatures adjust the stiffness of their membranes by changing the number of the rings between one to four per chain, leaving a constant distance determined by chains of 12 carbons between the ring stacks on the two sides of the monolayer membrane. The effect of perturbance of these stacks by straight chain lipids on the intermolecular interaction as revealed by the thermotropic properties was one of the objectives of this research.

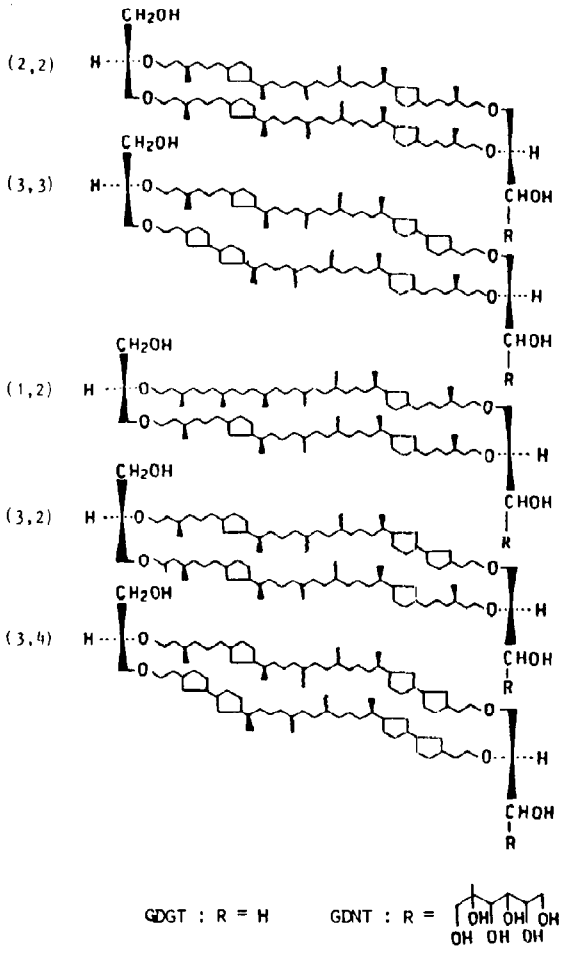

Fig. 1. Structures of isoprenoid tetraether backbone of complex lipids of *S. solfataricus*.

2. Experimental

2.1. Materials

The archaebacterium *S. solfataricus* was grown at 87°C. The lipids were extracted from the microorganisms with a Soxhlet apparatus for 12 h with chloroform/methanol (1 : 1, v/v). The total lipid extract was hydrolyzed with methanolic HCl. GDGT and GDNT were separated from the hydrolysis mixture by chromatography on a Silica gel column. $CHCl_3$/EtOH (9 : 1, v/v) eluted the GDGT fraction (≈ 17% of the lipids) and $CHCl_3$/MeOH (95 : 5, v/v) eluted the GDNT fraction (≈ 55% of the lipid). The chain composition of the two tetraether fractions was determined by HPLC. GDNT was analysed as fully acetylated derivative. HPLC was performed in *n*-hexane/ethyl acetate (6 : 4, v/v, for GDGT and 8 : 2, v/v, for acetylated GDNT) using a microporisil column (3.9 mm × 30 cm) with a flow rate of 0.5 ml/min. The molecular weights of the lipids are 1286–1294 and 1466–1472 for GDGT and of GDNT, respectively. The cyclopentane ring contents of the components in the GDGT and in the

GDNT mixtures were as follows:

GDGT: (2,2) ≈ 20%, (3,3) ≈ 29%, (2,1) ≈ 6%, (3,2) ≈ 41%, (3,4) ≈ 4%;
GDNT(I): (2,2) ≈ 18%, (3,2) ≈ 46%, (3,3) ≈ 30%, (3,4) ≈ 6%;
GDNT(II): (2,2) ≈ 22%, (3,2) ≈ 45%, (3,3) ≈ 32%, (3,4) ≈ 1%.

The chain composition of the lipids depends on the growth temperature [4] and seems to be very similar for GDGT and for GDNT except for the GDGT containing two and one cyclopentane ring chains. DPPC (chromatographically pure) was purchased from Dr. Berchtold's Laboratory, Bern, Switzerland.

2.2. Methods

The bacteriolipids or DPPC were dissolved in chloroform/methanol (2 : 1). Appropriate volumes of the two solutions were mixed, and the solvents were evaporated by a stream of nitrogen and subsequently kept under a vacuum of 0.1 Torr for 3 h in contact with a liquid nitrogen trap. The dry lipids were weighed into aluminum pans of the instrument, an excess (at least 4-fold) of a solution of 5×10^{-2} M KCl was added and the pans were sealed. In the experiments for determining the degree of hydration, the lipids and the salt solution were weighed before and after the experiment on a Cahn Electrobalance model 4100. In some cases the lipid solutions were evaporated directly in the aluminum pans, the lipid residue was weighed and aqueous solution added. The calorimetric measurements were performed on a Du Pont 990 differential scanning calorimeter with cell base II. The calibrated mode and scan rate of 5°C/min were used and the sensitivity as indicated in the figure legends. In each experiment the samples were rescanned several times till no difference between consecutive scans was detected.

For obtaining different degrees of hydration on the same sample the following procedure was adopted: A weighed quantity of water (≈ 18%, w/w, of the lipid) was positioned on the pan cover above the dry lipid (dried in high vacuum and with N_2 in the compartment of the Cahn balance). After sealing the pan, consecutive thermograms were run at two sensitivities of the instrument – the lower sensitivity giving the peak of melting of the free water and the higher one the peak of the melting of the lipid. During the scans the water distilled over and hydrated the lipid gradually, as indicated by the decrease of the size of the ice melting peak. The amount of free water was calculated from the ice melting peak and by subtracting this from the quantity of water added gave the degree of hydration of the lipid. Another procedure for obtaining partial hydration was followed by Gliozzi et al. [8] who gradually evaporated water from hydrated bipolar lipids in an open pan.

3. Results

3.1. Thermograms of pure bipolar lipids

In fig. 2, three thermograms of pure lipids in the presence of excess water are presented. Thermograms A and B are for two samples of GDNT (I and II) and thermogram C is for a sample of GDGT. The structure of the different lipids is given schematically in fig. 1. It is evident from fig. 2 that the thermograms of different batches of the same lipid are not completely identical even though the difference between curves A and B is exag-

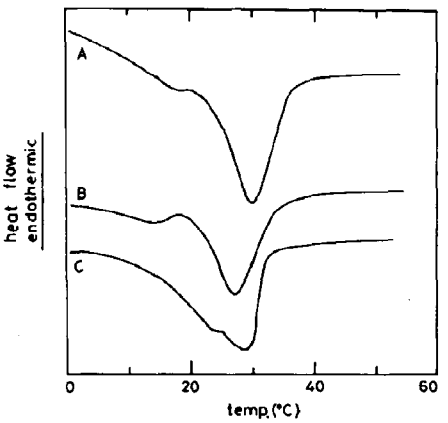

Fig. 2. Thermograms of the bipolar lipids in the presence of an excess of water. (A) GDNT I, 2.7 mg; (B) GDNT II, 1.8 mg; (C) GDGT, 1.4 mg. Sensitivity 0.02 mcal/s per inch.

gerated by the unequal weights and slopes. This is in agreement with the finding that minute variations in composition may have a pronounced effect on the thermograms [4], e.g., one cyclopentane ring per lipid molecule shifts the transition temperature of a dry lipid by about 20°C. As will be shown later, minute amounts of straight-chain lipids, e.g., DPPC, affect the thermograms even more. Thus, lipid contamination not exceeding 1% or slight variation in cyclopentane ring content can account for the differences in the thermograms. The thermograms of GDNT (traces A and B) are composed of two endotherms with midpoint temperatures of about 15 and 27°C whereas for GDGT (C) only one split peak with T_m at approx. 27°C is obtained. The shapes and temperatures of the peaks in the thermograms of the bipolar lipids, just like those of phosphoglycerides [9], sphingomyelins [10] or cerebrosides [11], depend very strongly on the degree of hydration. Fig. 3 presents the thermograms of dry GDNT and at different degrees of hydration obtained by consecutive water transfer as described in section 2. The dashed lines (lower sensitivity) represent the melting peaks of water. After reaching equilibrium the water transition peak changed no more and about 14% (w/w) of water corresponding to 13 H_2O molecules per lipid molecule became unfreezable. Hydration was probably not complete since the water melting peak is, as shown in fig. 3, below 0°C and the activity of the free water was considerably lower than 1 [11]. Nevertheless, the shape of the lipid peak is almost identical to that obtained in the presence of a large excess of water. The thermogram of the dry GDNT sample resembles that of a mixture of 4-GDNT with 5-GDNT as in ref. 7, however, the ratio of the areas of the low-temperature peak (13°C) to the high-temperature peak (47°C) is different: it is about 1.5 here, being about 1 in ref. 7. The difference may be caused by slight hydration but more probably by the different distribution of the cyclopentane rings on the two sides of the monolayer membrane. Increase in hydration reduces the size of the low-temperature peak and increases the high-temperature peak with a gradual shift toward lower temperature (27°C). Eventually, at a high degree of hydration only one peak accompanied by a small

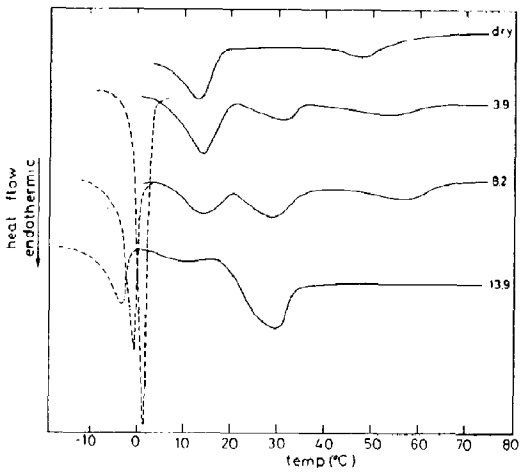

Fig. 3. Thermograms of dry GDNT II and at various degrees of hydration in wt% of H_2O of unfreezable water as indicated on the figure. Sensitivity 0.02 mcal/s per inch. (------) Melting of pure and of lipid-wetting but freezable water (distilled over after consecutive scans); sensitivity 0.1 mcal/s per inch.

shoulder remains. X-ray measurements carried out by Luzati (A. Gliozzi, personal communication) indicated that in the dry or in the partly hydrated state GDNT undergoes above 35°C a transition from the lamellar to other polymorphic crystalline structures. The peak around 50°C seems to correspond to these transitions. The two transition peaks below 30°C may result from the asymmetric configuration of GDNT with the glycerol residues on one side and the nonitols on the other side with the adjacent cyclopentane rings on either side of the monolayer. Such an arrangement might cause a difference in the enthalpy of melting of the two sides of the molecule which is expressed in the appearance of two endotherms in the thermogram. Hydration may partly randomize the orientation of the head groups producing one transition peak at intermediate temperatures. On the other hand, for a symmetric molecule, GDGT, only one melting peak is obtained in either the hydrated or nonhydrated state (ref. 8 and this work). It does not show polymorphism and it retains its lamellar structure through all the temperatures and degrees of hydration.

3.2. Mixtures of bacteriolipid with DPPC

3.2.1. GDNT/DPPC

Thermograms of mixtures of GDNT I with DPPC at different weight ratios are given in fig. 4a. Thermograms of mixtures of GDNT II gave similar behavior, differing only in small details. The interesting feature of these thermograms is the large effect of very small amounts of added DPPC. At a concentration of 9% (w/w) DPPC the peak at 27°C disappears and the small peak at approx. 15°C is strongly enhanced. In fig. 4b the gradual change of the shape of the thermograms between 3 and 9% DPPC is shown. The molecular weight of the bipolar GDNT is about twice that of DPPC or the weight per polar group is about the same. Thus, one DPPC seems to disturb completely the structure of about 10–12 neighboring half-molecules of GDNT. The disturbance is in the direction of recovery of the low-temperature peak prevailing in the nonhydrated (dry) GDNT while the high-temperature peak vanishes. The behavior of the mixtures of DPPC and bacteriolipid differs completely from the thermotropic properties of other lipid mixtures [13]. Mixtures of two miscible lipids differing in transition temperature render transition peaks at temperatures in between those of the pure components, whereas blends of immiscible or partly miscible lipids retain, albeit modified, their individual transition peaks. Mixtures of GDNT with DPPC do not fit any of the two cases. The downward shift of the melting temperature and disappearance of the second peak in bacteriolipid upon addition of small concentrations of DPPC indicates not only that the DPPC molecules are separated from each other but that the single molecules serve merely as spacers with respect to GDNT. This behavior also demonstrates itself in the change of the melting enthalpy with composition (fig. 5). Up to 10% added DPPC, there is no increase and there may be even a slight decrease in the enthalpy of melting per unit weight of the lipid. Only above this concentration of DPPC in the lipid mixture does the enthalpy start increasing (in parallel with the line giving the average enthalpy of the components). As seen from fig. 4a, concurrently a shoulder at around 25°C starts appearing on the thermograms. The shoulder increases in size and its temperature is shifted upwards with addition of DPPC, up to about 40%. At 47.5% DPPC the low- and high-temperature peaks merge into one peak with T_m =

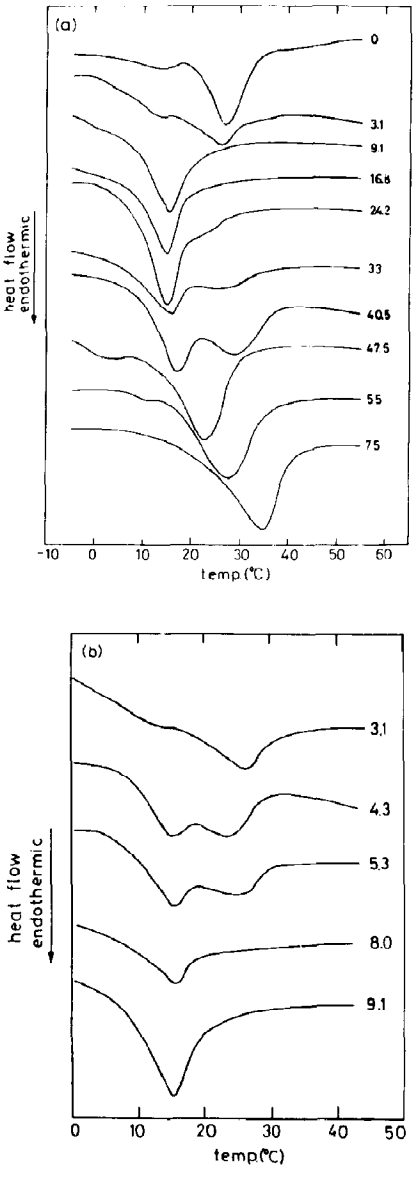

Fig. 4. Thermograms of GDNT II/DPPC mixtures in the presence of an excess of water. DPPC added (% w/w) is indicated on the figure. (a) Whole concentration range of DPPC, (b) low concentration range of DPPC.

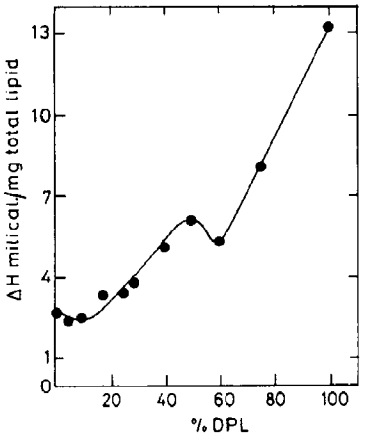

Fig. 5. Enthalpy of melting of GDNT II/DPPC mixtures as a function of % DPPC added.

22.5°C. The T_m of this peak rises with further increase in the DPPC content of the lipid mixture. In the region where the two peaks merge (around 50% DPPC) a minimum in the enthalpy of melting is observed (fig. 5). Further increase in DPPC concentration causes a steep increase of ΔH. This behavior can also be explained by DPPC-induced segregation of the two head groups with formation of regions of different melting properties. If DPPC prefers one head group, say, nonitol over the glycerol diether, at low concentrations it will serve as a spacer to the cyclopentane rings on this side decreasing the interactions between the rings and only the other side will undergo phase transition. If this is the case, at 8–9% DPPC it is surrounded by five to six nonitol half-molecules. At increasing concentrations (up to 40% DPPC) it continues accumulating on the nonitol side, with the evolution of the second peak and with gradual increase in ΔH and shift of its T_m to higher temperatures. Above a certain threshold concentration randomization of the bipolar lipid head group and DPPC molecule distribution occurs, and the two peaks merge into one with a decrease of the total enthalpy. The minimum in the enthalpy is caused by their entering as spacers between the cyclopentane rings at the second head group region.

3.2.2. GDGT / DPPC

When dry GDGT is heated for the first time (after being kept at low temperature) a split peak is obtained between 22.5 and 28.5°C; upon rescanning the splitting disappears and only one sharp peak at 25°C is obtained. In the presence of water, a split peak is obtained with maxima at 25 and 29°C (fig. 6). This behavior suggests segregation into sides richer and poorer in cyclopentane rings. Addition of up to 9% of DPPC to GDGT does not influence the lower temperature (24°C) transition peak while the 29°C peak vanishes. Further addition of DPPC seems to cause formation of domains containing DPPC/GDGT mixtures at two compositions undergoing transition at two temperatures about 5°C apart and always below 25°C. Only above 25% DPPC added does a peak start to appear at 29°C. The size and temperature of this peak increase with DPPC content of the lipid mixture. Up to 25–30% DPPC the enthalpy of melting of the mixtures is lower than the sum of the enthalpies of the pure components (fig. 7) (per unit weight of the lipid) in spite of the higher enthalpy of the added DPPC as compared to that

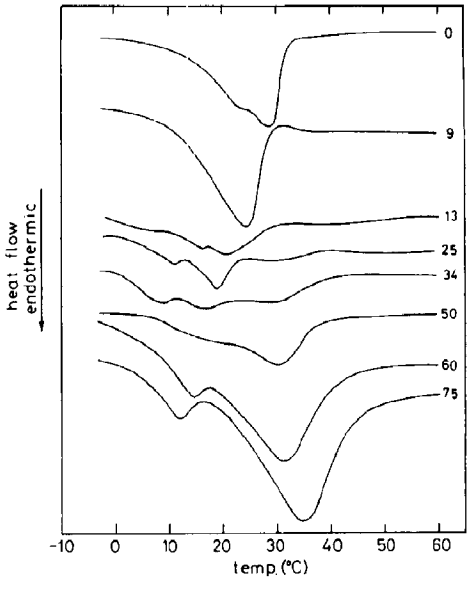

Fig. 6. Thermograms of GDGT/DPPC mixtures in the presence of an excess of water. DPPC (% w/w) is indicated on the figure.

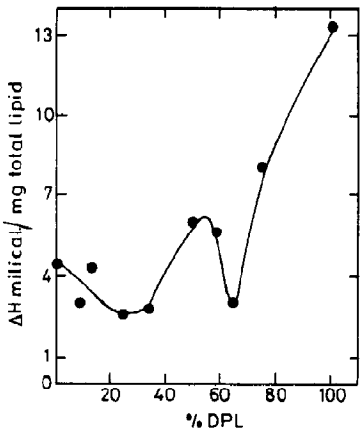

Fig. 7. Enthalpy of melting of GDGT/DPPC mixtures as a function of % DPPC added.

of GDGT. Above 30% DPPC the enthalpy per unit weight increases till about 50% DPPC, it drops to a minimum at 64% DPPC rising again towards the enthalpy of pure DPPC. It seems that DPPC up to about 50% segregates the lipid into different domains with enhanced hydration, suppressing the transition enthalpy. The maximal suppression of enthalpy occurs around 50% (w/w) DPPC. Above this content of DPPC the higher transition enthalpies of the DPPC-rich domains raise the average enthalpy level. Around 60% DPPC merging of the peaks with concomitant lowering of the transition enthalpy occurs. Upon adding DPPC above this molar ratio almost linear increase of the specific transition enthalpy is observed till the value of pure DPPC is obtained.

3.3. Lipid hydration

The unfreezable strongly bound water was determined by subtracting the freezable water (as obtained from the melting peak) from the added water (18–25%, w/w, with respect to the total lipid). Thus, the determination of the unfreezable water could be accurate only within ±15% for GDNT and ±25% for GDGT. GDNT prevents 12 ± 1.8% of its weight of water from freezing whereas GDGT renders only about 4% of water unfreezable. Assuming an average molecular weight of 1470 for GDNT and 1290 for GDGT, this corresponds to 10 ± 1.5 water molecules per GDNT and 2.8 ± 0.7 per GDGT molecule. These calculations show that each hydroxyl group either of nonitol or of glycerol binds between 1.1 and 1.5 water molecules. The amount of bound or unfreezable water increases gradually with subsequent addition of DPPC to the lipid mixture up to 17% for pure DPPC.

4. Discussion

The thermotropic behavior of the bipolar lipids depends strongly on the structure of the molecules, degree of hydration and the degree of purity of the sample. In the dry state [4] the symmetrical molecule GDGT renders one endothermic peak after rescanning, whereas in the thermograms of the asymmetric GDNT two endothermic peaks are seen. It is concluded from this behavior that in the dry and in partially hydrated GDNT orientation of the bipolar molecules occurs, with different head groups to either side producing regions differing in thermotropic properties. Addition of water to GDNT seems to randomize the orientation of the molecules producing one transition peak with temperature of melting intermediate between those of the dry state. Hydration of GDGT shifts the peak from 24 to about 29°C (not shown, see also ref. 8), however, a shoulder at 24°C remains indicating that nonhydrated domains would be retained even in the presence of a large excess of water. This suggests that hydration is a cooperative process resulting in formation of different hydration domains when the hydration is not complete or when the degree of hydration changes in the course of heating. This is in line with the gradual disappearance and evolving of transition peaks when increasing the degree of hydration of GDNT as observed in fig. 3. The water of hydration affecting lipid phase transition was assumed to be at least in two distinctly different states: the unfreezable one [14,15] and the solvating router reduced in activity. The concept of unfreezable water is a phenomenological one. Its identification with bound water is in keeping with the BET (Brunauer, Emmett, Teller adsorp-

tion isotherm) of water adsorption by egg phosphatidylcholine (PC) [16], a molecule of which adsorbs 2.5 H_2O molecules in the first layer and 5 molecules in the second one. The adsorption in the two layers corresponds to the number of H_2O molecules rendered unfreezable by one PC molecule. However, the unfreezable water could still freeze as its activity is gradually decreasing [17], producing a very broad practically undetectable transition peak [12].

The increase in transition temperature with hydration observed in GDGT and in one peak of GDNT is quite exceptional. Usually the transition temperature decreases with hydration [9–11]. We suggest that hydrogen bonds between the neighboring glycerols impose a twist on the chains interfering with effective stacking of the cyclopentane rings. Hydration breaks the tight intermolecular hydrogen bonds and relaxes the strain on the hydrocarbon chains allowing optimal stacking of the rings. The tightly stacked cyclopentane rings do not allow intercalation of dye molecules, e.g., 8-anilino-1-naphthalenesulfonic acid which therefore does not exhibit any increase of quantum yield in the presence of these lipids [18].

The interaction of added DPPC with the bipolar lipids also seems to be a cooperative phenomenon. When added to GDNT it has a derandomizing effect since it prefers one of the head groups forming cooperative domains. We suggest that it prefers the nonitol. This suggestion is based on the experimental finding that when DPPC is added either to GDGT or GDNT peaks below 20°C start evolving. These peaks are assumed to correspond to lipid chain transitions in the vicinity of the same head group and therefore the glycerol ether. The lowering of T_m of this region is assumed to result from the strain imposed by the DPPC intercalated between the molecules on the other side of the bipolar monolayer.

This cooperative segregation of the interaction domains is in agreement with the finding that in vesicles formed from GDNT/DPPC lipid mixture DPPC accumulates on the outer side of the vesicles [18]. Between 8 and 9% (w/w) of DPPC in the DPPC/GDNT mixture (fig. 4a) the peak at 28°C disappears completely and the peak at 15°C is fully evolved. Since the molecular weight of GDNT is twice that of DPPC, one DPPC molecule is surrounded at this point by five to six bipolar molecules presumably on the nonitol side of the bipolar monolayer. Up to this concentration of DPPC the total enthalpy does not increase; with further addition of DPPC up to 20% (1 DPPC per 2 GDNT) the same single peak is retained but the total enthalpy increases. Only above this concentration of DPPC added does another domain with a transition peak at higher temperature start forming. The 15°C transition peak is retained almost till the molar ratio of 2 DPPC/GDNT is reached when the two domains merge giving one transition peak with a lowering of the total enthalpy of melting. With further addition of DPPC the transition temperature and the transition enthalpy increase nearly linearly toward the value of pure DPPC. Similar behavior is observed with GDGT, only here the enthalpy minimum is obtained at approx. 62% (w/w) DPPC or at a ratio of nearly 3 molecules DPPC per GDGT molecule. If we assume that the enthalpy minima occurring at 2 : 1 and 3 : 1 correspond to complexes of DPPC with GDNT and with GDGT, respectively, then the lipid mixtures at higher concentrations of DPPC can be considered as ideal cosolutions of these complexes with DPPC.

Acknowledgement

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