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Physicochemical characterization of tetraether lipids from *Thermoplasma acidophilum*. V. Evidence for the existence of a metastable state in lipids with acyclic hydrocarbon chains

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The main glycophospholipid of *Thermoplasma acidophilum*, grown at 39 °C, is composed of a di-isopranol-2,3-glycero-tetraether. It has been characterized in hydrated systems by calorimetry. Unlike its equivalent grown at 59 °C, it shows complex phase properties, which include at least three different phases, (1) a liquid-analogue state (C), which is stable above 20 °C, (2) a metastable solid-analogue state (A) formed by supercooling of the liquid-analogue state (C) and (3) a stable solid-analogue state (B), which is slowly formed and may include a close chain packing of lipids and a network of hydrogen bonds between the headgroups. A high fraction of acyclic isopranol chains seems to be a prerequisite for the formation of state (B). A phase diagram, displaying the observed states and the transitions between them is proposed.

1. Introduction

Poikilothermal organisms such as bacteria show a pronounced modulation of the fatty acid composition of their membrane lipids according to their growth temperature, yielding an increased fraction of unsaturated, shorter or branched chain fatty acids at reduced growth temperature [1]. By this process lipid membranes are maintained in the fluid state and the micro-environment of the membrane (including its microviscosity) is kept constant, a prerequisite for proper function of membrane enzymes [2,3].

Bacteria usually show a lipid phase transition (gel-to-liquid crystalline) at about 20 K below growth temperature [4], which is linked to a strongly enhanced permeability of the membrane for alkali ions and polyols. If this event occurs in living cells, it leads to cell death [5]. These phase transitions are coupled to a thermal enthalpy change (ΔH), which can be monitored by calorimetry.

The correlation between the physicochemical properties of membrane lipids and the growth temperature of its source organism was studied using the main glycophospholipid of *Thermoplasma acidophilum*, a thermoacidophilic archaebacterium which grows between 39 °C and 59 °C at about pH = 1. Its basic membrane lipid structure was characterized by Langworthy [6] as consisting of a di-isopropanol-2,3-glycero-tetraether, modulated by the attachment of different headgroups at both 1-positions of the two glycerols. The main glycero-phospholipid was identified as containing a glucose at one end and a phosphatic acid esterified to another glycerol at the other end [7]. In response to adapting of the organism to higher growth temperatures, the C_{40}-biphytanyl chains show up to two pentane cyclizations, reducing the rotational freedom of the chains and increasing their rigidity. The main glycophospholipid (MPL) was isolated and purified by chromatographic means [6], and that of organisms cultivated at 59 °C (designated as MPL59) has already been characterized by calorimetry [8,9]. It showed no change in its heat capacity between 0 and 70 °C and only a small endothermic transition ($\Delta H = 14$ kJ/mol) was found at sub-zero temperatures (−30 °C → −5 °C). In spite of the anionic character of the lipid, this transition was almost independent of pH or the presence of divalent cations. In addition a glass transition was found at temperatures between −90 °C and −50 °C. In this paper the main glyco-phospholipid from *Thermoplasma acidophilum* cultivated at 39 °C (designated at MPL39) is characterized by calorimetry and compared with that

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of the organism grown at 59 °C (MPL59). These data throw light on the molecular behaviour of this unique type of lipid, exhibiting at least two different solid-analogue states.

2. Materials and Methods

Thermoplasma acidophilum was grown in Freundt's medium at 39 °C at pH = 1 under moderate aeration. Cells were harvested in the late exponential growth phase. Lipids were extracted from freeze-dried cells and the main glycophospholipid, designated as MPL39, purified to chromatographic purity, as checked by thin-layer chromatography with different solvent systems. The techniques of cell cultivation and lipid purification have been described in detail elsewhere [8]. MPL39 was used in all experiments described here.

Differential thermoanalysis was performed with a Mettler TA3000/DSC30 instrument equipped with a liquid nitrogen cooling device. Heating and cooling scans were run repetitively at rates between dT/dt = 0.02 and 0.14 K/s. Samples contained 3–6 mg lipid plus 20 µl buffer (400 mmol/l sodium cacodylate/HCl, 12.5 mol/l ethyleneglycol, pH = 7.0). Details of the calorimetry are given in Ref. 8. All chemicals were analytical grade and organic solvents of 'Resi' quality (Baker Inc.).

3. Results

Fig. 1 depicts a series of thermograms of the main tetraetherlipid of *Thermoplasma acidophilum* grown at 39 °C. The cooling scan from 60 °C to −50 °C at a rate of 0.08 K/s (Fig. 1e) showed a single phase transition between approximately −25 °C and −45 °C, which, as explained below, was denoted (C → A). Subsequent heating curves of the sample were complex, comprising at least two endotherms and an exotherm between −30 °C and 20 °C with heat rate dependent properties (Fig. 1 curves a–d); between 25 °C and 75 °C no further changes in the heat capacity of the sample were observed. In response to increased heating rates (Fig. 1, curves a–d) the heat flow rate between sample and DTA-calorimeter, and therefore the amplitudes of the peaks, of course increased. Yet the enthalpy changes ΔH (peak area per heating rate) varied with heating rate as depicted in Fig. 2. ΔH of the low-temperature endotherm (A → C) at −28 °C to −15 °C increased with heating rate up to a scan rate of 0.08 K/s, however ΔH value of the exotherm (A/C → B′) and the second endotherm (B′ → C) decreased steadily. By extrapolation to $dT/dt \to 0$ values of ΔH(B′ → C) = 69 kC/mol and ΔH(A/C → B′) = 49 kJ/mol were obtained.

The modulations of ΔH on the heating rate were interpreted as indications of a kinetic process involved in the transformation between at least three different

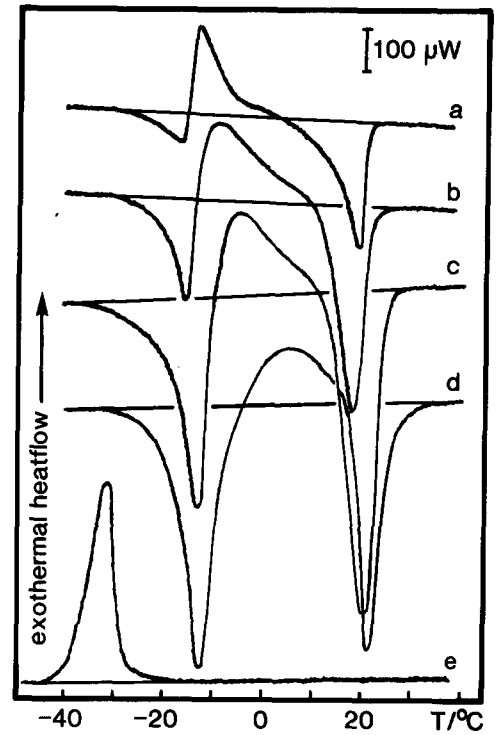

Fig. 1. DTA scans of the main glycophospholipid of *Thermoplasma acidophilum* cultivated at 39 °C (MPL39). Heating scans with (a) dT/dt = 0.02 K/s, (b) dT/dt = 0.04 K/s, (c) dT/dt = 0.08 K/s, (d) dT/dt = 0.13 K/s. (e) Cooling scan with dT/dt = −0.08 K/s.

lipid states which, analogous those observed by Boggs et al. in cerebroside sulfates [10], were denoted (A), (B) and (C). This assumption was confirmed in heating scans starting from various temperatures (Fig. 3). Curve

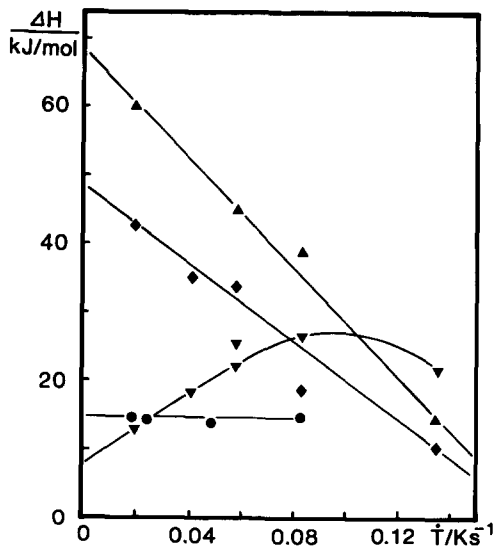

Fig. 2. Enthalpy change (ΔH) of the observed phase transitions of MPL39 and MPL59 as a function of the heating rate dT/dt. ▲ MPL39, melting endotherm (B′ → C) around T_m = 20 °C; ◆ MPL39, exotherm (A/C → B′) around 0 °C; ▼ MPL39, endotherm (A → C) around T_m = −15 °C; ● MPL59, endotherm (A → C) around T_m = −10 °C.

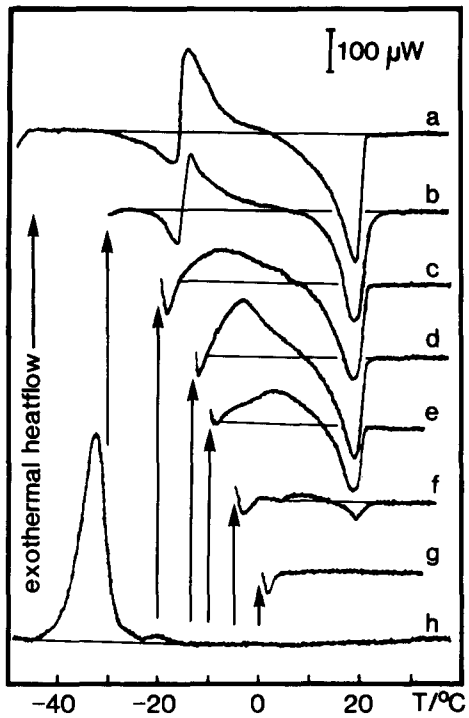

Fig. 3. DTA cooling scan ($dT/dt = -0.08$ K/s) and a series of heating curves ($dT/dt = 0.02$ K/s) after cooling ($dT/dt = -0.08$ K/s) to the starting temperature indicated by an arrow.

(3a) shows the typical cooling curve of MPL39 which has already been described. When a cooling scan was terminated at the temperature indicated by arrows (Fig. 3, curves b–h) and a heating curve (scan rate $dT/dt = 0.02$ K/s) run two minutes thereafter, a set of heating scans was obtained which showed a simultaneous appearance of the exotherm (A/C → B′) and the high temperature endotherm (B′ → C). Due to the thermal non-equilibrium in the DTA-calorimeter, heatflow rate data were not reliable for the first 2–3 min of a scan (equivalent to 3 K in the experiment depicted in Fig. 3), so that a precise comparison of their ΔH values was not possible. Yet the heat content of endotherm and exotherm proved to fall in the same range for all curves of Fig. 3. Thus the endothermic phase transition (B′ → C) was found only when it was preceded by the exotherm (A/C → B′) and both ΔH values increased in value as the onset temperature of the heating scan was lowered.

The nature of these transitions was further substantiated by the observations summarized in Fig. 4. Curve (4a) presents the cooling curve of MPL39, showing a phase transition between −35 °C and −55 °C (C → A) and a 'glass transition' between −55 °C and −83 °C. Curve (4b) represents a heating curve commenced immediately after the completion of the cooling scan, showing the typical sequence of thermal events already described (Fig. 1, curve e) (plus the preceding 'glass transition'). If, however, the heating scan was stopped at 0 °C (i.e. during the exothermal reaction A/C → B′) and the sample kept at that temperature for 10 min to allow the completion of this reaction, before being cooled down again to −100 °C, the exothermal transition (C → A) in the cooling scan around −45 °C disappeared (Fig. 4, curve c). Moreover, in the subsequent heating scan (Fig. 4, curve d) the ΔH values of the endothermal transition around −20 °C (A → C) and the following exothermal transition at 0 °C (A/C → B′) were reduced by at least two orders of magnitude. Only an endotherm at $T_m = 21$ °C (B → C) was observed. Its heat flow as a function of temperature proved to be very similar to the high-temperature transition (B′ → C) described earlier, yet its transition temperature was 2 K higher and $\Delta H = 60$ kJ/mol. Therefore this phase (B) was distinguished from the former one (B′). This result indicates that during heating scans ($dT/dt = 0.02$ K/s) the time of sweeping over the exothermal transition (A/C → B′) around 0 °C was not sufficient for the complete formation of a stable gel state (B). Upon cooling, neither (B) nor (B′) transformed back into phase (A), so that phase (B/B′) was considered as stable (at low temperatures), whereas (A) was metastable; kinetic barriers partly prevented a transformation (A → B).

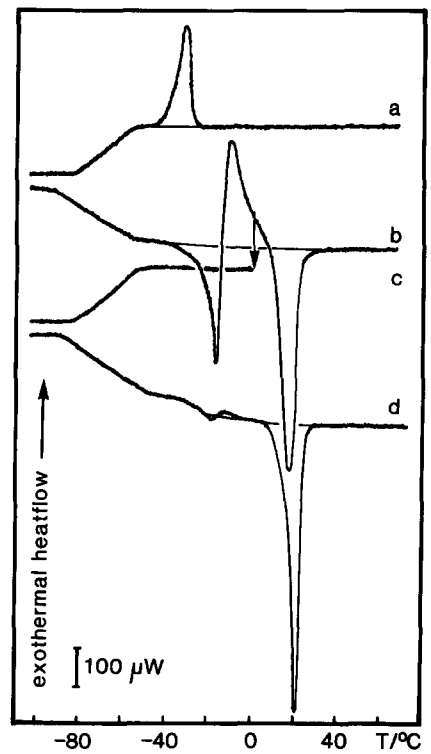

Fig. 4. DTA heating and cooling scans of MPL39 as a function of the pre-incubation conditions of the sample ($dT/dt = 0.06$ K/s). (a) cooling scan from 60 °C; (b) heating scan from −100 °C to 60 °C; (c) cooling scan after the termination of a heating scan at 0 °C and isothermal incubation of the sample at this temperature for 10 min in order to allow completion of the exothermal reaction; (d) heating curve following cooling curve (c).

When liquid-analogue lipid (C) was cooled down from 60 °C to 6 °C and stored at that temperature for several days, a transition (B″ → C) was monitored between 0 and 20 °C, whose heat flow as a function of temperature was very similar to that of the solid-analogue state (B′) (T_m = 21 °C); however, its melting enthalpy increased with incubation time at 6 °C up to a maximal value of −53 kJ/mol which amounted only to 75% of the value found from the extrapolation of ΔH in Fig. 2 to $dT/dt \rightarrow 0$ (data not shown). Because of the significant difference in ΔH and T_m, this state (B″) was distinguished from the phases (B) and (B′).

The time constants of the transitions were roughly estimated from heating and cooling curves at different scan rates (Fig. 1). The time period between the temperature of maximal heat flow of the melting endotherm of state (A) and its offset by the exothermal formation of state (B′) amounted to 2–3 min irrespective of the scan rate. Thus the time constant for the formation of the stable phase (B′) from a melting phase (A) was estimated to be around 3 min. The formation of (B″) by supercooling of liquid-analogue lipid (C) at 6 °C, on the other hand, was in the order of 100 h and the transition from the liquid-analogue state (C) to the metastable solid-analogue state (A) in the order of minutes at T = −25 °C (Fig. 2).

4. Discussion

The thermotropic characteristics of MPL39 can be rationalized in the phase model depicted in Fig. 5 by postulating four different phases:

(i) A liquid-analogue phase (C) which is stable at temperatures above 20 °C.

(ii) A metastable solid-analogue phase (A), which was obtained by fast cooling (0.08 K/s) of the liquid-analogue lipid (C) in an exothermal transition between −20 °C and −45 °C. (The transition C → A also takes place isothermally at temperatures between −5 °C and −30 °C with a temperature-dependent time constant. This quasi-stable state (A) melts in a broad transition between −30 °C and −5 °C, about 18 K above the transition temperature observed during cooling and thus with a pronounced hysteresis.)

(iii) A stable solid-analogue phase (B), exothermally formed upon (endothermal) melting of solid-analogue lipid (A → C). (The heatflows of the endothermal melting of (A) and the exothermal recrystallization of melted lipid into (B′) overlap between −10 °C and 10 °C and partially offset each other. The time constant of its formation was estimated to be in the order of several minutes, so even at moderate heating rates the formation of (B) is incomplete, resulting in a phase (B′) with reduced T_m and ΔH. The formation of (B)/(B′) is irreversible, i.e., lipid does not directly transform back into state (A), and therefore it is denoted 'stable'. (B) melts at 18–21 °C and is transformed into the liquid-analogue phase (C). Upon extended supercooling at 6 °C, (C) is slowly transformed into state (B″) which has a melting curve very similar to that of (B), however with a reduced ΔH value. (B″) is also believed to be a defective form of (B).)

(iv) A sub-phase, separated from (A) and (B/B′) by a glass transition between −50 °C and −83 °C.

The relative expression of the three thermal peaks is determined by the heating rate of a scan, the time constant of the transitions and their mutual offset, resulting in a curve of net heat flow between sample and calorimeter.

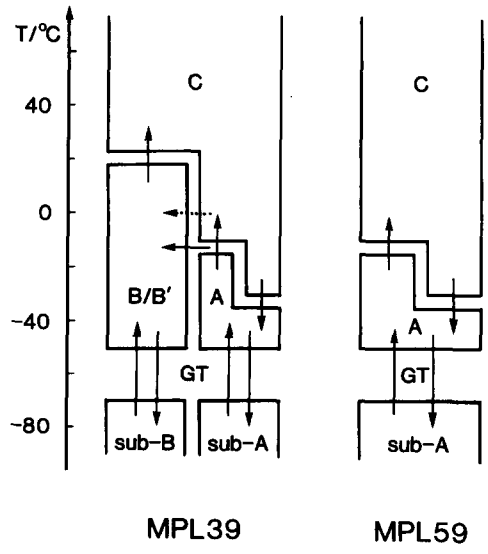

Fig. 5. Proposed phase model for MPL39 and MPL59.

These thermotropic properties of MPL39 differ significantly from those of the corresponding main phospholipid of *Thermoplasma acidophilum* cells grown at 59 °C (MPL59) which heating scans only showed a single phase transition with T_m = −10 °C, i.e., in the same temperature range as that of the metastable solid analogue phase of MPL39 (A → C). Thus it is assumed that MPL59 exists in the phases (A), (C) and subphase only. The enthalpy change of MPL59 amounted to −14 kJ/mol, which is about half of that of MPL39. Moreover, no indication for the occurrence of a metastability was observed in MPL59 under the experimental conditions used. As reported by Langworthy and co-workers [7], the only structural difference between MPL39 and MPL59 is the replacement of methylbranches by cyclopentane moieties, which may have substantial consequences in respect to its ability to form regularly packed, condensed phases. Langworthy determined the fraction of acyclic hydrocarbon chains of the main tetraether-lipid of *Thermoplasma acidophilum* as a function of growth temperature to be 62% at a growth temperature of 39 °C (MPL39) and 26% at 59 °C (MPL59). Thus the melting enthalpy ΔH(A → C) proved

to be proportional to the fraction of acyclic lipid within the experimental limits. This correlation may be fortuitous, but it may also indicate that the acyclic fraction alone is able to form phase (B) (possibly by undergoing a phase separation). Under this assumption, the ΔH value for pure acyclic lipid would (by extrapolation) be around 50 kJ/mol. Taking into consideration the membrane-spanning structure of the lipid macrocycle, it would be comparable to $\Delta H = 25$ kJ/mol for 'normal' bipolar lipids. Still, this value is considerably higher than that of lipids with methyl-branched fatty acids of comparable chain length [11]. The difference may result from the membrane-spanning structure of the tetraether lipid with a polar headgroup on each end of the macrocycle being anchored in the lipid-water interphase so that only those conformations of the hydrocarbon chain are permissible which do not significantly shorten the length of the macrocycle. Moreover, as both hydrocarbon chains are covalently linked to the same glycerol molecules at head and tail, only those patterns of motion are permissible, which alter the length of both hydrocarbon chains synchronously. In contrast, bipolar lipids are not subject to these constraints in the thermal movement of their hydrocarbon chains and already the presence of isomethylbranched fatty acids leads to a high degree of disorder in the hydrophobic region [11]. The low-temperature endotherm around $-15\,^{\circ}$C therefore can be interpreted as the melting of an ordered state of the fraction of acyclic chains (phase separation) or of the whole lipid system induced by the fraction of acyclic lipid.

The complex phase polymorphism of MPL39 described here is not a unique feature of tetraether lipids; similar phenomena have also been reported in hydrated samples of phosphatidylcholines (subtransition) [12–14], lecithins with short isobranched fatty acids [11,15], phosphatidylglycerols (high temperature transition after extensive supercooling) [16] and subtransition [16,17], phosphatidylethanolamines [18–22] and their derivatives [23,24], phosphatidylserines [25], glycosyldiglycerides [26–28], sphingomyelins [29–33], cerebrosides [31,34–41] and cerebroside sulfates [10,42–44]. In addition they are found in phosphatidylglycerols in the presence of divalent cations [45–47] and in mixed lipid systems [46]. In the latter case the exothermal transition may represent a phase separation. All these systems are characterized by a slow transition from the metastable to the stable solid-analogue state with time constants between minutes [10,41] and months [16].

In most of these systems there is evidence that the establishment of intermolecular hydrogen bonds is involved in the formation of the stable high-melting state [48], which includes the closer packing and reduced hydration of lipid headgroups, resulting in an increase in the melting temperature by 10–30 K [48]. The highest values were found in lipids where electrostatic repulsion of the headgroup charges did not play a significant role. Similar properties can be expected for MPL39 as the chemical components of its headgroups (glycerol, glucose, phosphate) have the potential for establishing intermolecular H-bonds and as the number of phosphate groups is limited to one per tetraether macrocycle, corresponding to an average of 0.5 phosphates per headgroup. The isoprene hydrocarbon chains may also require more space than unbranched hydrocarbon chains, possibly resulting in an increased distance between adjacent phosphate groups (and in their natural environment of pH = 1 the phosphate will probably be protonated anyway). The observed increase in the melting temperature (from $-10\,^{\circ}$C to $20\,^{\circ}$C) of 30 K is at the higher end of that observed in other lipids, but still in line with the general rule for the formation of intermolecular H-bonds between lipid headgroups.

The extremely high melting enthalpy of these lipids of 40–80 kJ/mol [10,16,29,31,48] is another indication of the involvement of an H-bond network, as their melting (thermal movement of the hydrocarbon chains) leads to a lateral expansion of the lipid, an interruption of the network of hydrogen bonds and an increased hydration of the headgroups. Similar thermotropic properties have been reported here for the stable solid-analogue state (B′) of MPL39 ($\Delta H = 68$ kJ/mol). If this value is related to the fraction of acyclic lipid (62%) alone, the enthalpy change for pure acyclic lipid would amount to $\Delta H = 108$ kJ/mol, corresponding to $\Delta H = 54$ kJ/mol for half a macrocycle which is comparable to a bipolar lipid. However it is not known if phase separation of acyclic lipid occurs under those conditions or if a common phase together with cyclized MPL39 lipids is maintained (with a reduced concentration of hydrogen bonds).

The observed kinetic effects support the assumption of the formation of an H-bond network. *N*-Lignoceryl-galactosylsphingosine had already entered into the stable phase during the cooling cycle [40], whereas C24 : 0h-cerebroside sulfates [10] (and MPL39) were kinetically inhibited and did not transform until they were reheated. At slow heating rates the latter lipids melted endothermally, followed by an exothermal recrystallization to a crystalline state with a very high degree of order, high melting temperature and melting enthalpy [10,40,43]. At higher heating rates ($dT/dt = 0.08$ K/s), however, the formation of the stable phase was incomplete with a reduced T_m and ΔH [10,43]. A complete formation of the stable phase could only be obtained by isothermal incubation at the temperature of the exothermal reaction [10,40]. The time constant of this transition was determined to be 3.5 min in stearoylcerebroside sulfate [43].

A direct transformation from the liquid-analogue state to the stable solid-analogue state was also observed in palmitoylgalactosphingosine about 10 K be-

low T_m [31]; its enthalpy change, however, amounted only to 73% of the maximal value obtained via the melting of the metastable solid-analogue state (compare also C18 : 0 cerebroside sulfate [10]). This reduction in ΔH was explained by an elevated residue of hydrate between headgroups [31]. Similar results have been reported here for MPL39. As the headgroups of sphingomyelin, cerebroside and cerebroside sulfate are comparable to that of the tetraether lipid MPL39, similar hydration processes can be expected and a thermotropic phase behaviour of similar complexity is not surprising.

A high fraction of acyclic hydrocarbon chains seems to be a precondition for the formation of an ordered state of macrocyclic lipid which may include a network of hydrogen bonds between its headgroups. A sufficiently close packing of regularly branched hydrocarbon chains can be gained by an alternating shift of the hydrocarbon chains along their main axis (formation of an alternating mesh) or a tilting of hydrocarbon chains from the surface normality as proposed by Blaurock for diphytanylglyceroglucosine [49]. In both models the distance between adjacent lipid molecules is reduced and the potential for a polar interaction between headgroups is increased.

Does phase separation occur in MPL39? And is close packing of the acyclic fraction in the stable solid-analogue state (B) realized by an intermesh chain packing and/or the formation of a network of hydrogen bonds between headgroups? These questions cannot be answered from calorimetric data alone. They require investigations by X-ray diffraction, Raman spectroscopy and pressure-surface diagrams of all three phases described as well as studies of the methyl ether derivatives of the main glycophospholipid MPL39 which would not be expected to be able to form such a network of hydrogen bonds and therefore should be lacking in phase (B).

In summary, it has been shown that MPL39 follows the general pattern, that lipids from microorganisms show a lipid phase transition around 20 K below the growth temperature of its source organism. However, the stable solid-analogue state (B), which possibly includes the formation of intermolecular H-bonds between the headgroups of acyclic lipids, is only obtained under special conditions, including a precooling to subzero temperatures. In lipids from *Thermoplasma acidophilum* grown at 59 °C, however, similar properties were not found. It is possible that MPL59 shows a similar phase transition at higher temperatures (around 40 °C), but that the right conditions for its transformation have not yet been found (presence of antifreeze and high lipid concentration). Its formation may have been inhibited by a higher kinetic barrier or completely prevented by the presence of cyclopentane moieties. The low melting point and the kinetic inhibition of its formation may be a consequence of the extreme ecological living conditions of *T. acidophilum* which requires a high degree of versatility for survival.

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