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PHYSICOCHEMICAL CHARACTERIZATION OF TETRAETHER LIPIDS FROM *THERMOPLASMA ACIDOPHILUM*

DIFFERENTIAL SCANNING CALORIMETRY STUDIES ON GLYCOLIPIDS AND GLYCOPHOSPHOLIPIDS

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Thermoplasma acidophilum **is an obligate thermoacidophilic archaebacterium, which was grown semi-continuously at 59°C and pH 2. From freeze-dried cells, three lipid fractions were isolated: the total lipid mixture, the glycolipid fraction, and the glycophospholipid fraction, each comprising several components. By chromatographic means, the major lipid was isolated from the glycophospholipid fraction and purified to chromatographic homogeneity. The basic structure of the polar lipids is a large, symmetrical macrocycle made up of two saturated, methyl-branched C_{40} hydrocarbon chains linked to two glycerol molecules by ether bonds. Most likely the tetraether lipids span the *Thermoplasma acidophilum* membrane thus forming a monomolecular apolar core. As shown by differential scanning calorimetry these lipids are in a liquid-crystalline state over an unusually wide temperature range, as compared with other bacterial membrane lipids. Between 0°C and 80°C, no indication for a thermotropic phase transition was found. In the range between −5°C and −30°C all polar lipids exhibit a transition with an enthalpy change being by about one order of magnitude lower than that of bilayer-forming ester phospholipids. Between −50°C and −95°C, all lipids pass a glass-transition. Addition of calcium or magnesium ions or of protons in molar excess had only minor influence on the phase transition of fraction of the glycophospholipid fraction. We therefore conclude that the thermotropic properties of the polar tetraether lipids of *Thermoplasma acidophilum* are mainly determined by their apolar moieties.**

Introduction

Thermoplasma acidophilum is a thermoacidophilic archaebacterium isolated by Darland et al. [1] from a steaming, self-heated coal refuse pile. It grows optimally at 59°C and pH 2. The more or less spherical cells are devoid of peptidoglycans and S-layers, normally present in bacteria. Thus, the only barrier between the cellular interior and the environment is a 4–5 nm thick plasma membrane, which exhibits an unusual chemical and physical stability [2]. It is composed of approximately (by weight) 60% protein, 10% carbohydrate, and 30% lipid. The latter exhibits a peculiar basic structure first described by Langworthy and collaborators [3]. As illustrated in Fig. 1, it is composed of two repetitively methyl-branched, saturated C_{40} hydrocarbon chains, lin-

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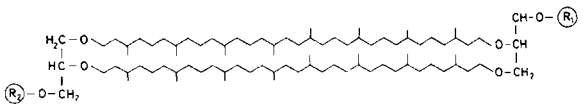

Fig. 1. Basic structure of the dialkyldiglyceroltetraethers from *Thermoplasma acidophilum* [13]. Glycolipids: R_1 = H, R_2 = one or several carbohydrates. Glycophospholipids: R_1 = carbohydrate, R_2 = phosphorylglycerol. MPL: R_1 = unidentified carbohydrate, R_2 = phosphorylglycerol.

ked to two glycerol moieties by ether bonds. Structurally, the hydrocarbon chains are derived from two phytanol residues covalently linked head-to-head. Thus, a symmetrical, stretched macrocyclic tetraether molecule is formed which is resistent to acid hydrolysis and autoxidation. The length of the molecule amounts to approx. 4 nm thus corresponding to the width of the apolar core of the membrane. In response to alteration of the growth temperature, the structure of the macrocycle is modulated by intramolecular cyclisations leading to hydrocarbon chains with up to four cyclopentane moieties [4]. By attachment of different polar residues to one or both of the free OH-groups of the glycerol moieties, a large variety of derivatives is generated. The residues consist of one or several hexose units, or phosphoric acid esterified with carbohydrates. Thus, the membrane lipids can be divided into two classes: uncharged glycolipids, and glycophospholipids which are monovalent anions over a wide pH range. Furthermore, a lipopolysaccharide was described the headgroup of which is formed by an unbranched chain of 24 hexose units [5,6].

As yet, little is known about the physicochemical properties of tetraether lipids and the molecular organization of archaebacterial membranes. The peculiar basic structure of the tetraether molecules suggest that the membranes are organized as monolayers instead of the bilayer structures present in all other types of cells and organelles. Such an assumption was first formulated on the basis of observations which had shown that the plasma membranes of *Thermoplasma acidophilum* could not be freeze-fractured within the apolar plane (Verkleij, A., personal communication, see also Ref. 7). Recently, Gliozzi and co-workers [8] reported that nonitol tetraether lipids from *Sulfolobus solfataricus*, which are structurally closely related to the diglycerol tetraether lipids from *Thermoplasma acidophilum*, form black lipid films which are indeed monolayers.

In a previous paper we have shown that polar lipids derived from *Thermoplasma acidophilum* easily form stable liposomes; the permeability of the liposomal membranes for low molecular solutes, e.g. polyols, is extremely low as compared to that of artificial membranes derived from bilayer-forming phospholipids such as phosphatidylcholines [9]. In the present communication we describe calorimetry studies performed with various lipid preparations from *Thermoplasma acidophilum*. The results show that these lipids are in a liquid-crystalline state over a wide temperature range. Furthermore, the data strongly suggest that the thermotropic properties of the hydrated tetraether lipids are only little influenced by the chemical nature and the degree of dissociation of the polar headgroups.

Methods and Materials

Organism and culture conditions. Thermoplasma acidophilum was kindly supplied by Dr. Zillig, Max-Planck-Institut für Biochemie, München, F.R.G. Cultures were grown in Freundt medium [10] at 59°C and at pH 2 under moderate aeration in a fermenter 'Biostat S' (Braun AG, Melsungen, F.R.G.). The growth was continuously monitored by means of a photometric device. Cells were harvested in the late exponential phase ($A_{578\,nm}$ approx. 1) by centrifugation and washed three times with distilled water. The average yield was 400 mg dry cells per litre of culture fluid.

Extraction and purification of lipids. Lipids were extracted from freeze-dried cells and subsequently separated into the three main fractions: apolar lipids, glycolipids and glycophospholipids, following the procedure described by Langworthy and collaborators [11]. From the glycophospholipid fraction, the major lipid was isolated by silicic acid chromatography using a chloroform-methanol gradient. The purity of the preparation was checked by thin-layer chromatography using the solvent systems published by Langworthy and co-workers [3].

Differential scanning calorimetry. DSC was performed by means of a Mettler TA 3000/DSC 30

instrument equipped with a liquid nitrogen cooling device (Mettler Instrumente AG, Greifensee, Switzerland). Scans were run at rates of $dT/dt = 0.02–0.06\ K \cdot s^{-1}$ Baseline fluctuations amounted to $P = \pm 10\ \mu W$ yielding a detection limit of about $\Delta H \cdot m^{-1} = 0.5\ J \cdot g^{-1}$ (typical peak width of 20 K). Because of uncertainties in the molar masses of the various lipid preparations, enthalpy changes were referred to lipid mass instead of molarity. For sample preparations, 3–4 mg lipid dissolved in chloroform/methanol (2 : 1, v/v) were added into DSC pans (aluminium, $V = 40\ \mu l$) in fractions of 5 μl and dried under a stream of nitrogen. After evaporation under vacuum for at least 16 h, lipid mass was gravimetrically determined ($\Delta m = \pm 10\ \mu g$); 20 μl buffer was then added, pans were sealed and kept at 60°C for 24 h to complete hydration and equilibration. Reference pans containing 20 μl buffer were handled in the same manner. The buffer normally contained 200 mM NaCl and 380 mM cacodylate-HCl; the pH was adjusted to 6.8. Cacodylate was chosen as buffer in order to avoid pH shifts in response to cooling to temperatures $\ll 0$°C [12]. For some experiments, 200 mM of $MgCl_2$ or $CaCl_2$ were added to the buffer solution, and/or the pH was adjusted to 2.0 using 0.1 M HCl. If not stated otherwise, all buffers contained 12.5 M ethylene glycol as antifreeze, guaranteeing a freeze point below −100°C. It is important to mention that the concentration and the chemical nature of the antifreeze significantly influence the transition temperatures of the samples and therefore should be kept constant within each set of measurements.

Chemicals. All chemicals used were analytical grade reagents. Organic solvents purchased from Baker Inc. were of 'Resi' quality. Dipalmitoylphosphatidylglycerol was obtained from Sigma Chemical Corporation, München, F.R.G.

Results

Fig. 2 shows DSC scans of four different lipid fractions isolated from *Thermoplasma acidophilum*: the total lipid extract, the glycolipid fraction, the glycophospholipid fraction, and the main component of the latter. With exception of the lipopolysaccharide, the total lipid fraction comprises all polar and apolar lipids of the cells, including

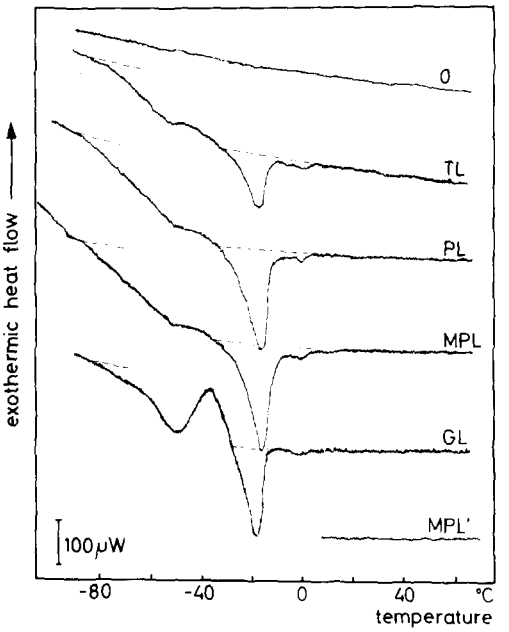

Fig. 2. Thermograms (heating curves) of different lipid fractions isolated from *Thermoplasma acidophilum*. O, control (blank) scans; TL, total lipid extract; PL, glycophospholipid fraction; MPL, main component of fraction PL; GL, glycolipid fraction. The heating rate was 0.06 $K \cdot s^{-1}$; the buffer (380 mM sodium cacodylate-HCl/200 mM NaCl (pH 7)) contained 12.5 M ethylene glycol. MPL' was run in the absence of ethylene glycol.

biosynthetic precursors [11]. The glycolipids, glycophospholipids, and the main glycophospholipid are tetraether lipids. The glycolipid fraction is composed of ten, and the glycophospholipid fraction of nine different components, whereas the main glycophospholipid is a single, chromatographically pure lipid which has been described as the glycerol phosphoryl-glycosyl derivative of the tetraether [13]. The basic structure is shown in Fig. 1. The main glycophospholipid is the quantitatively dominating lipid in *Thermoplasma acidophilum* membranes, making up approximately (by weight) 50% of the total lipid and 75% of the glycophospholipid fraction [13].

In spite of the large differences in the structure and polarity of their polar headgroups, the thermotropic properties of the various lipid fractions are apparently very similar. Within the limits of detection of the instrument ($\Delta H \cdot m^{-1} = 0.5\ J \cdot g^{-1}$), no indication for the occurrence of thermo-

tropic phase transitions in the range between 5°C and 65°C can be detected. The same result was obtained when the measurements were performed using sample preparations without antifreeze. To validate these findings, control measurements (data not shown) were made with a Privalov differential scanning calorimeter, the sensitivity of which is about one order of magnitude higher than that of the Mettler instrument. Even under these conditions we failed to observe any phase transition of the glycophospholipid fraction in the range of 5°C (the lower limit of the instrument) to 100°C.

At temperatures below 0°C, all the four fractions show a small enthalpy change (Table I). The heat contents of these changes are low, being by about one order of magnitude lower than those usually obtained with bilayer-forming phospholipids, e.g., phosphatidylcholines [14]. The upper and lower temperature limits of the transitions, as well as the positions of the peak minima are very similar in the total lipid, glycophospholipid and main glycophospholipid preparation. Furthermore, the glycophospholipid fraction and the main glycophospholipid exhibit ΔH values that are indistinguishable from each other. Thus, the thermotropic behaviour of the main component can be taken as reflecting the behaviour of the whole glycophospholipid fraction.

TABLE I

PHYSICOCHEMICAL DATA ON THE THERMOTROPIC PHASE TRANSITIONS OF DIFFERENT LIPIDS FROM *THERMOPLASMA ACIDOPHILUM*

t_m: transition temperature (temperature at the peak minimum); t_l, t_u: lower (t_l) and upper (t_u) temperature limits of the transitions. Buffer: 380 mM cacodylate/HCl (pH 6.8)/200 mM NaCl/12.5 M ethylene glycol. The absolute values of the transition temperatures are slightly dependent on the nature and concentration of the antifreeze present in the samples. The values listed in the table were obtained from a set of measurements performed under the same conditions.

Lipid fraction	t_m (°C)	ΔH (J/g)	t_l (°C)	t_u (°C)
Total lipid	−16.5	5.0	−40	−10
Glycophospholipid	−14.3	8.9	−34	−8
Main glycophospholipid	−14.0	8.9	−32	−7
Glycolipid	−17.7	9.0	−30	−13

The ΔH value of the total lipid mixture is significantly lower than that of the other two fractions. This can be attributed to the transition-quenching effect of apolar lipids present in this fraction. The transitions of the glycophospholipid fraction and the main glycophospholipid are quenched quantitatively to the same extent, when the apolar lipids from the total lipid fraction are added in physiological range (data not shown).

In comparison to the above mentioned fractions, the glycolipid fraction exhibits a more complex thermotropic behaviour. Firstly, there is a small shift in the temperature range at which the phase transition occurs; the magnitude of the enthalpy change, however, is similar to those of the other fractions. Secondly, between −65°C and −30°C, the glycolipid fraction passes a metastable state. The sum of the positive and negative enthalpy changes is zero. As illustrated in Fig. 2, metastable states were not detected in the glycophospholipid fraction and its main component, nor in the total lipid fraction, although the latter comprises all components of the glycolipid fraction too. Thus, constituents of the total lipid fraction prevent the occurrence of this state.

As reflected by the deviation from the extrapolated base lines at $t < -50$°C, all lipids undergo a glass transition which is terminated at about −95°C. This phenomenon will be described in more detail in a separate communication.

The preceding data suggest that the nature of the polar headgroups have only minor effects on the thermotropic phase behaviour of the tetraether lipids from *Thermoplasma acidophilum*. This assumption is confirmed by the data summarized in Fig. 3. In these experiments, the polarity of the headgroups was modulated by (a) lowering the pH of the incubation buffer from 6.8 to 2.0, a value beyond the pK of the phosphoryl residue, and (b) by addition of calcium or magnesium ions in molar excess. The results show that neither protons nor the divalent cations substantially influence the transition range or the melting enthalpies. Under same experimental conditions, the transition temperature of another anionic phospholipid, dipalmitoylphosphatidylglycerol, which, however, forms bilayers, is increased by about 25°C upon lowering the pH from 7 to 2, and by 48°C upon addition of calcium ions (Fig. 4). The ΔH values

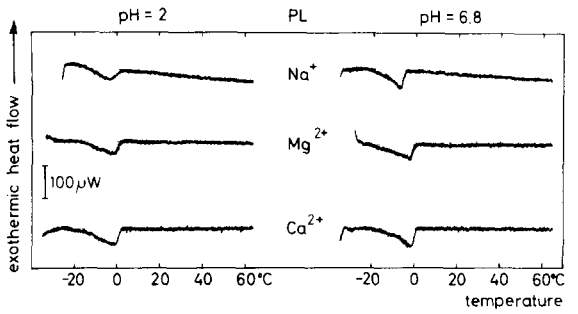

Fig. 3. Effect of protons and divalent cations on the thermotrophic properties of the glycophospholipids from *Thermoplasma acidophilum*. Scans (heating curves) were run at a rate of 0.02 $K \cdot s^{-1}$. The basic buffer (380 mM sodium acodylate-HCl) contained 8.9 M ethylene glycol and, as additivities, 200 mM NaCl, $CaCl_2$ or $MgCl_2$, as indicated.

increased from 37 to 46.6 $kJ \cdot mol^{-1}$ (addition of H^+) and from 37 to 72.5 $kJ \cdot mol^{-1}$ (addition of calcium ions), respectively. These values are in accordance with data published by Van Dijck et al. [14].

A comparison between the transition temperatures reported in Fig. 2 and Table I, respectively, and Fig. 3 (pH 6.8; 200 mM NaCl as the control scan) reveals a difference of about 7 K. This is mainly due to the differences in the concentrations of ethylene glycol in the preparations. As will be shown in a separate paper the chemical nature and the concentration of the antifreeze significantly influence the transition temperatures.

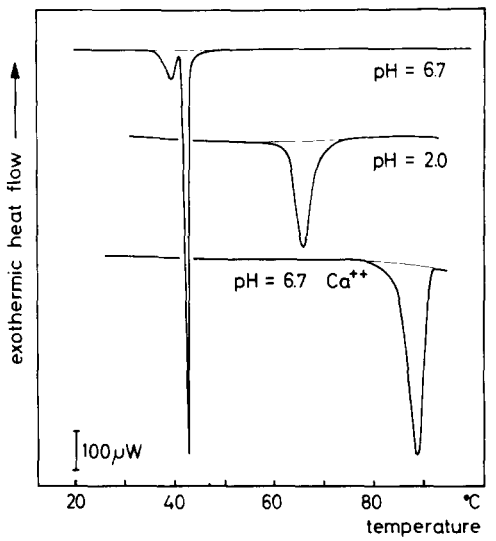

Fig. 4. Effect of protons and calcium ions on the thermotropic properties of dipalmitoylphosphatidylglycerol. Scans (heating curves) were run at a rate of 0.017 $K \cdot s^{-1}$. The composition of the antifreeze-free buffer was the following: (a) pH 6.7: 380 mM sodium cacodylate-HCl, 1 M NaCl ±47 mM $CaCl_2$, corresponding to a molar ratio of calcium ions/phospholipid = 0.7; (b) pH 2.0: 370 mM glycine-HCl, 1 M NaCl.

Discussion

Membrane lipids of growing bacteria and other cells are preserved (via adaptional processes) in a liquid-crystalline state which is required to maintain various membrane functions. In many bacteria, at temperatures few degrees below the growth temperature, the membrane lipids begin to pass from the fluid-crystalline to the solid-gel state. Since most of the membrane lipids are chemically heterogeneous with respect to both hydrocarbon chains and polar headgroups, the width of the phase transition is rather broad, comprising 30°C or even more.

As a reliable method for detecting phase transitions, DSC has been widely employed [14–16]. The main advantage of this technique is the possibility to obtain quantitative information, i.e., to describe the transition in thermodynamic terms, as well as the fact that, in contrast to EPR or fluorescence technique probes are not required, the distribution of which in the apolar phase remains unclear, and which may disturb just those intermolecular interactions which are subjected to analysis. The main disadvantage lies in the fact that the sensitivity of most of the instruments is not sufficient to allow studies of lipid phase transitions in situ, i.e., in the intact membrane, with appropriate accuracy. Since the amount of lipid present in the *Thermoplasma* membrane is approx. 25% (by weight) at least 15 mg (dry mass) of pure membranes would be required to study thermal lipid properties in situ. This exceeds by far the capacity of the pans available, the total volume of which is in the order of 40 μl. Another, genuine limitation of the method is given by the fact that DSC detects only bulk lipid phase transitions. This disadvantage, however, becomes irrelevant, if, as in some of the experiments reported in this paper, chemically homogeneous material is studied such as the purified main glycophospholipid.

The DSC data we obtained from hydrated samples of various lipid preparations from *Thermoplasma acidophilum*, grown at 59°C, show:

(a) All lipids exhibit an unusual high degree of fluidity. We were unable to detect any phase transition at $t > 0$°C. This finding is consistent with film balance experiments and permeability studies on liposomes prepared from the same batches as used for the DSC studies described here (unpublished data). A phase transition could be observed with all the lipid preparations between -30°C and -5°C. Thus, different to eubacterial lipids, the membrane lipids of *Thermoplasma acidophilum* remain in a fluid-crystalline state over a wide temperature range below the growth temperature. The enthalpy changes are small as compared with those of bilayer-forming neutral or anionic phospholipids. The low values can be explained by the assumption that the large number of methyl branches in the apolar moiety of the tetraether molecules hinders the formation of regular, lattice-like structures even at $t < 0$°C. The same explanation may be true for the broadness of the transition range in the case of the purified main glycophospholipid, indicating a low degree of cooperativity between the single molecules.

(b) The thermotropic properties of the *Thermoplasma acidophilum* phospholipids are only slightly influenced by the ionic environment. Neither lowering the pH from 6.8 to 2, a value at which most of the phosphoryl residues have lost their free charge, nor addition of divalent cations, which are known to electrostatically interact with the anionic groups and to shift the transitions of anionic, bilayer-forming phospholipids to higher temperatures [14,17] exhibited substantial effects on the phase transition of the tetraether phospholipid of *Thermoplasma acidophilum*. We therefore conclude that the phase behaviour of these lipids is predominantly determined by their apolar moieties. This assumption is supported by the observation that, at pH 6.8, no significant differences in the calorimetric behaviour of the glycolipid fraction, the glycophospholipid fraction, and the main glycophospholipid were detected, with exception of a metastability observed at very low temperatures in the glycolipid fraction. Thus, considerable differences in the physicochemical properties of 'conventional' bilayer-forming polar lipids and the membrane-spanning, monolayer-forming tetraether lipids from *Thermoplasma acidophilum* must be stated.

Some of our data do not agree with findings published by Haug and collaborators on the thermotropic behaviour of *Thermoplasma acidophilum* membranes as monitored by EPR. In plots of the hyperfine splitting parameter, $2T_{\parallel}$, versus temperature, Vierstra and Haug [18] found discontinuities at 15.3°C and 35.6°C which they attributed to lipid phase transitions. Yang and Haug [19] described phase transitions at 16°C and 43°C, whereas Strong and Haug [20] observed phase transitions at 21°C and 57°C. Irrespective of the diversity of the data, it has to be taken into consideration that these authors used membranes and lipid preparations which contained significant amounts of bilayer-forming phospholipids with unbranched fatty acid residues, i.e., phosphatidylcholine, phosphatidylserine, and phosphatidylethanolamine [21]. In contrast to this but in accordance with data from Langworthy and collaborators [11,13] our lipid preparations are free of such ester lipids. Moreover, it has to be considered that the basic structure of the spin label used by Haug and coworkers, 5-nitroxy-stearate, is an unbranched C_{18} fatty acid. Thus, the discrepancy between our and Haug's observations may be explained by the assumption that the spin label, due to its molecular shape, is preferentially enriched in domains containing mainly bilayer-forming lipids. If it should be integrated into membrane-spanning bulk tetraether lipid phases it inevitably monitors disturbed intermolecular interactions in its surrounding. On the other hand, the ESR probe may partition also into non-bulk tetraether lipid domains which are induced by non-lipid membrane constituents, e.g. proteins. These special microstructures may give rise to temperature discontinuities, which can be detected in intact membranes, but not in pure lipid preparations. Lastly we cannot exclude that the discontinuities in the plots of $2T_{\parallel}$ versus temperature indicate temperature-induced alterations in the molecular organization of lipids or conformational changes which are not related to enthalpy changes.

In a recent paper, Gliozzi and collaborators [22] reported the occurrence of thermotropic phase transitions in a tetraether lipid isolated from

another archaebacterium, *Sulfolobus solfataricus*. While the enthalpy changes were comparable to those described in this paper, the transition range was shifted by about 30°C to higher temperatures. This difference may be attributed to the fact that the lipids used by Gliozzi and co-workers, were subjected to methanolysis and therefore had lost their polar headgroups. The polarity of the residual two OH-groups at the ends of the tetraether, however, is presumably far below the minimum required for the formation of ordered monomolecular layers in aqueous environment. Such assumption is supported by the observation that tetraether lipids after methanolytic treatment had lost their ability to form stable black lipid membranes [22] and that non-hydrated samples exhibited very similar calorimetric properties as hydrated material [22]. Thus, the molecular organization of the lipids in aqueous environment is unclear. In contrast to this, the lipids used in our studies do form stable black lipid membranes (Bamberg, E., personal communication) and also form large unilamellar liposomes which are stable for many days. Therefore, the studies reported by Gliozzi et al. and by us were dealing with two different materials, a group of highly polar lipids containing all polar headgroups, and a lipid preparatoin of very low polarity.

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