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PURIFICATION AND CHARACTERIZATION OF A LIPOSOMAL-FORMING TETRAETHER LIPID FRACTION

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Summary Polar lipid E, PLE, a native tetraether lipid mixture from *Sulfolobus acidocaldarius* is shown to spontaneously form multilamellar liposomes in aqueous media. PLE lipids were isolated as a single fraction from the crude lipid extract of *S. acidocaldarius* on a reverse-phase column followed by TLC and methanol precipitation. The methanol-precipitated mixture was able to form liposomes, whereas the non-precipitated material did not. It has thus been demonstrated, for the first time, that tetraether lipids from *S. acidocaldarius* can form liposomes, *per se* , in aqueous media. © 1990 Academic Press, Inc.

Bipolar, tetraether lipids constitute the major lipids of thermoacidophilic archaebacteria (1). These thermally stable lipids possess several unique structural features including bipolarity, ether bonds, and highly-branched biphytanyl chains. The tetraether lipids are believed to be able to form, by virtue of their structure, monomolecular membranes (2). It is of interest to determine what the structure/property relationships are for liposomes formed these highly unusual lipids, addressing, in particular, issues of membrane morphology and stability. We report here on a liposomal-forming tetraether and its freeze-fracture characteristics.

In a previous paper (3), we described the purification of the hydrolyzed tetraether lipid glycerol dialkyl nonitol tetraether (GDNT). GDNT and glycerol dialkyl glycerol tetraether (GDGT) make up the two major classes of hydrolyzed tetraether lipids from *Sulfolobus acidocaldarius* , a thermoacidophilic archaebacteria. Polar lipid E[1] is a major polar lipid fraction from *S. acidocaldarius* and consists of a mixture of tetraether lipids with either a GDGT or GDNT backbone and phosphatidylmyoinositol as one polar moiety and either glucopyranose or galactopyranosyl glucopyranose disaccaride as the other polar group (Fig. 1).

Previous purification procedures for the tetraether native lipids required elution from silica gel columns with different ratios of chloroform and methanol (4, 5) . Lipids purified in such a

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[1] We follow Langworthy's nomenclature in this paper. Another often-used name for PLE is P2; see, for example, Gulik, et al (9).

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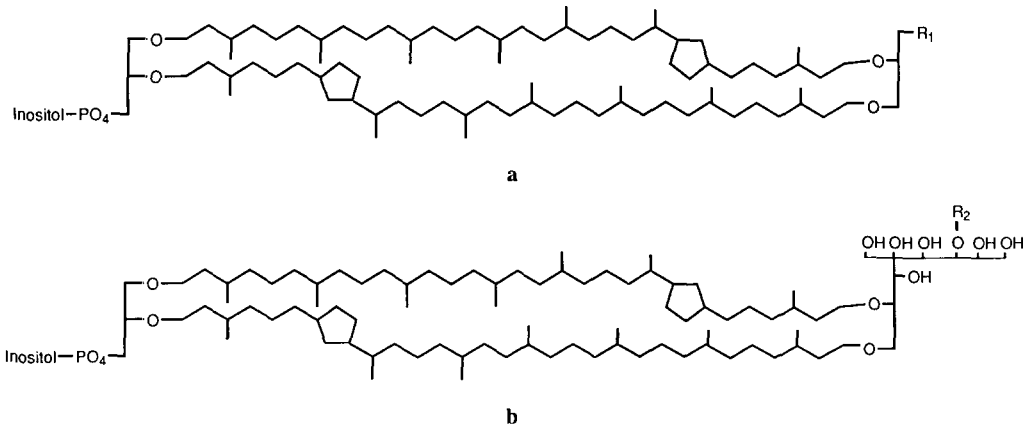

Figure 1. The structures of the lipid mixture PLE consist of (a) the GDGT skeleton with phosphatidylmyoinositol on one end and R_1 = β-D-glucopyranose and (b) the GDNT skeleton with phosphatidylmyoinositol on one end and R_2 = β-D-galactopyranosyl-β-D-glucopyranose. The number of cyclopentane rings can vary from 0 to 8 (5).

manner often contain difficult-to-remove silica gel particles due to the presence of the methanol which solubilizes a certain amount of silica gel. We present here a method for the purification of PLE using a reverse-phase coulumn and solvent-precipitation to overcome the problem of contamination by silica gel. Interestingly, only after the last step, methanol-precipitation, do the lipids form an aqueous dispersion. This is the first time that any lipids from *S. acidocaldarius* has been shown to form liposomes and constitutes a necessary first step in the study of how the unique chemical structures of these tetraether lipids affect their membrane properties and stability.

MATERIALS AND METHODS

S. acidocaldarius (ATCC, strain 33909, Rockville, MD) were grown at 65-67°C in 20-L glass carbuoys containing 15 L of ATCC medium 1256 supplemented with 1 g/L glucose (6). Air was bubbled through the aqueous medium at 1 L/min and growth was monitored by absorbance at 420 and 540 nm. Cells were harvested by centrifugation at the onset of stationary phase, lyophilized, and stored at -20 °C until used. Average yield was 300-400 mg dry cells/L culture.

Crude lipids were Soxhlet-extracted with chloroform-methanol (1:1), as described previously (3). Ten to fifteen mg of the crude lipid extract were suspended in 2 mL of methanol-water (1:1) with the help of a bath sonicator (Sonicor, Copiague, NY). The suspension was transferred to a Prep Sep C18 extraction column (Fisher Scientific, Fair Lawn, NJ). The column was eluted with 20 mL each of the following solvents: methanol-water (1:1), chloroform-methanol-water (0.8:2:0.8), and chloroform-methanol-water 65:25:4). The chloroform-methanol-water (0.8:2:0.8) fraction, containing most of the PLE, was dried under vacuum, weighed (20% w/w recovery), and redissolved in a minimum amount of chloroform-methanol-water (65:25:4) with the help of a bath sonicator. Ten mg of lipid were spotted on a LK5 TLC plate (Whatman, Hillsboro, OR). The plate was dried under a hood for 30 min and then developed with chloroform-methanol-water (65:25:4). A wide band corresponding to PLE (Rf = 0.25) was located by exposure to iodine vapor, scraped, and eluted with chloroform-methanol-water (1:2:0.8). The eluent was filtered through a Millex HV filter (Millipore, Bedford, MA). After drying the filtrate under vacuum, the residue was suspended in a minimum amount of chloroform-methanol-water (65:25:4) with the help of a bath sonicator. To precipitate the lipids, five volumes of methanol were added to the suspension at room temperature and the precipitate collected by centrifugation. Final yield was 4% of initial crude lipid extract.

Phosphate was identified by molybdenum spray (7) on TLC plate after developing in chloroform-methanol-water (65:25:4). GDGT, GDNT, and the polar head groups were identified by hydrolyzing the PLE lipids for 18 h at 70 °C with 1 N methanol-HCl. Chloroform and water were added to the hydrolysate in the ratio of chloroform-methanol-water (8:4:3) to form a two-phase system. The lower phase was dried and spotted on TLC (Kiesel 60, EM Science, Cherry Hill, NJ) along with standards (1) using chloroform-methanol (10:0.5) as the developing solvent (Rf of GDNT = 0.7, GDGT = 0.12). The upper phase was dried and acylated with acetic anhydride in the presence of pyridine at 60 °C for 6 h. The acetates were analyzed by HPTLC (EM Science, Cherry Hill, NJ) along with appropriate standards using hexane-diethyl ether (3:7) as developing solvent (Rf of galactose acetate = 0.38, glucose acetate = 0.31, inositol acetate 0.25).

Vibrational characterization was performed on a Perkin-Elmer 1800 fourier transform IR (FTIR) Spectrophotometer controlled by a PE Series 7000 computer. PLE spectrum was taken as a dried film on BaF_2 windows. Resolution was 2 cm^{-1}.

Test tubes were prepared with 1.8 mg of PLE each. The lipid was initially dried with argon and then overnight in a vacuum oven. 1 mL of distilled water (pH 6.5) or HEPES buffer (pH 7.0), at concentrations of 0.05, 0.1, 0.2, 0.5, and 1 M were introduced into each test tube.The aqueous samples were briefly warmed to 60 °C for 1/2 h and then allowed to stand overnight at room temperature. A vortexer was used to suspend any remaining lipids from the glass walls. pH was varied for the 0.1 M sample from 2.7 to 12.5.

Specimens for freeze-fracture were prepared with no cryoprotectants but were transferred directly to Balzer (Hudson, NH) copper specimen plates, equilibrated to room temperature, and quickly frozen by plunging into liquid propane. They were then transferred to a Balzer BAF 400D freeze-fracture device, fractured, and briefly etched for 30 s. Replicas were made at -100 °C and 10^{-6} Torr with a 2 nm Pt-C and a 20 nm carbon film. The replicas were floated off onto doubly-distilled water and transferred to sodium hypochlorite for 2 h, rinsed in doubly-distilled water and cleaned in 20% ethanol for 1 h. They were then picked up on 300 mesh copper grids. Electron micrographs were taken with a Zeiss EM10C (NYC, NY) transmission electron microscope.

RESULTS AND DISCUSSION

The PLE yield from the Prep Sep C18 column was 20 % (w/w) of the crude-lipids extract, which, after TLC and methanol precipitation, decreased to 4 %. The GDGT and GDNT backbone were identified by first hydrolyzing the lipids then spotting on TLC. Acetate derivatives of the sugars were used to confirm for the presence of glucose, galactose, and inositol. The FTIR spectrum exhibited major peaks corresponding to OH, CH_2 and CH_3 stretches and deformations, and C-O-C ether stretching vibrations consistent with the macrocyclic tetraether structure (3). In addition, phosphate bands at 1217 and 1076 cm^{-1} were evident.

Liposomal-formation studies showed immediate breakup of the dried lipid film from the walls of the test tubes when aqueous buffer or distilled water was introduced, although the breakup became less evident as the buffer concentration increased. Incubation at 60 °C appeared to speed the dispersing process. After standing in buffer overnight, only the 0.5 and 1.0 M buffer samples required further vortexing to completely disperse from the test tube walls. Changing the pH of a PLE suspension, made at neutral pH did not cause any precipitation to occur, although the suspension at pH 12.5 showed increased birefringence. Storage of the vesicles either at 4 °C or by freezing did not cause precipitation. The ability of PLE to disperse into aqueous suspension contrasts with GDNT and GDGT, which showed no tendency to spontaneously disperse in water solutions, even with sonication (3). GDNT can only be induced to suspend in aqueous medium by

injecting an acetone solution of the lipid into hot (≥ 75 °C) water of low salt concentration and neutral pH (8).

Freeze-fracture electron microscopy of the PLE suspensions showed typical multilamellar vesicle structures of about 1 μm in diameter (Fig.2). The freeze-fracture micrographs always exhibited cross-fracturing of the membranes. That is, there does not appear to be any midplane of a bilayer in these tetraether lipid membranes. This result, while not a definitive proof, is consistent with the hypothesis that the bipolar structure of the archaebacterial lipids should allow the lipids to form monomolecular membranes.

Even though tetraether lipids comprise 95% of the total lipids in *S. acidocaldarius* membranes, neither the hydrolyzed nor the native lipids have yet been reported to spontaneously form liposomes in aqueous media. Gulik, et al (9) reported, from x-ray data, that unsubstituted,

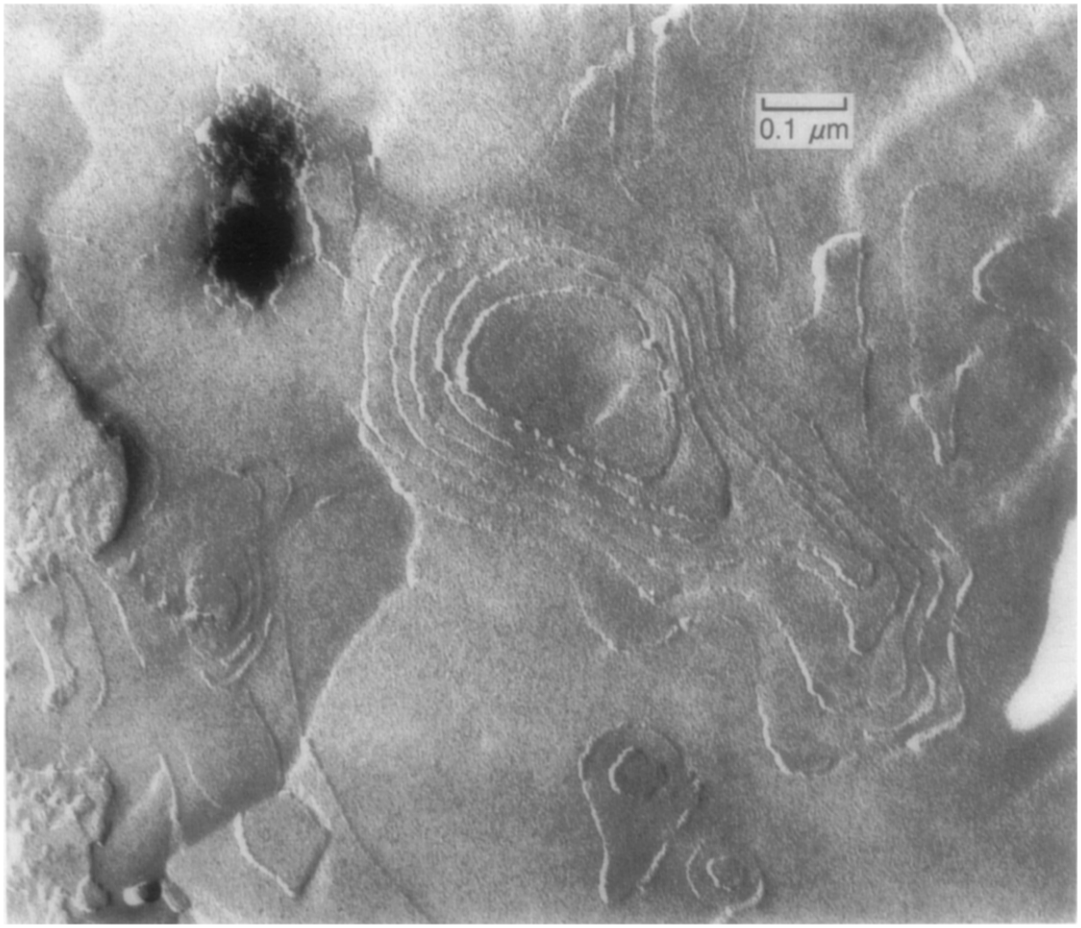

Figure 2. Freeze-fracture micrograph of PLE multilamellar liposomes prepared in 0.05 M HEPES buffer. Note the cross-fracture nature of the fracture plane.

or monosubstituted tetraether lipids from *S. acidocaldarius* do not form lamellar phases, while the disubstituted lipid fraction (PLE) exhibited lamellar phases throughout the known portions of its phase diagram[2]. There is one report on the formation of vesicles from mixtures of GDNT and egg phosphatidylcholine (EPC) with at least 25 mol % EPC (10)[3]. Mixtures with less than 25 mol % EPC did not form liposomes. However, the main polar lipid fraction (MPL) of the thermoacidophile, *Thermoplasma acidophilum* , has been reported to form liposome suspensions in dilute buffers (11,12). MPL is a glucosyl-phosphorylglycerol derivative of GDGT and bears some resemblance to the GDGT-derivative of PLE (Fig. 1a). Thus, the two types of tetraethers that have been reported to form liposomes both contain a negatively-charged group at one polar end and polar sugar or sugars at the other end.

In summary, we have purified PLE, a native polar tetraether lipid mixture from *S. acidocaldarius* , and have shown that this mixture will spontaneously form liposomes in aqueous media. These results extend the known range in which PLE exists in lamellar phase to temperatures below 45 °C.

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[2]The phase diagram for PLE (P2 in reference 9) only extended from 45 °C to ~85°C.

[3]Although Lelkes, et al (10) do not state which lipids of S. acidocaldarius were used, a later review article (13) gave GDNT as the lipid.

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