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Stability of diether $C_{25,25}$ liposomes from the hyperthermophilic archaeon *Aeropyrum pernix* K1

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ABSTRACT

Temperature and pH effects were studied for stability, structural organization, fluidity and permeability of vesicles from a polar lipid methanol fraction isolated from the *Aeropyrum pernix*. We determined the permeability of $C_{25,25}$ liposomes using fluorescence intensity of released calcein. At pH 7.0 and 9.0, and from 85 °C to 98 °C, only 10% of entrapped calcein was released. After 10 h at 90 °C, calcein release reached 27%, independent of pH. Fluorescence anisotropy measurements of hydrophobic probe 1,6-diphenyl-1,3,5-hexatriene revealed gradual changes up to 60 °C. At higher temperatures, the anisotropy did not change significantly. Fluorescence alone did not provide detailed and direct structural information about these $C_{25,25}$ liposomes, so we used electron paramagnetic resonance spectroscopy (EPR) and differential scanning calorimetry (DSC). From EPR spectra, mean membrane fluidity determined according to maximal hyperfine splitting and empirical correlation times showed continuous increases with temperature. Computer simulation of EPR spectra showed heterogeneous membranes of these $C_{25,25}$ liposomes: at low temperatures, they showed three types of membrane regions characterized by different motional modes. Above 65 °C, the membrane becomes homogeneous with only one fluid-like region. DSC thermograms of $C_{25,25}$ liposomes reveal a very broad and endothermic transition in the temperature range from 0 °C to 40 °C.

1. Introduction

The glycerol ether lipids are one of the most remarkable features that distinguish members of archaea from those of bacteria and eucarya (Woese et al., 1990). In bacteria and eucarya, the membrane phospholipids feature an *sn*-glycerol-3-phosphate scaffold, which is ester linked to the fatty acid (acyl) chains. In contrast, archaeal lipids use the opposite glycerol stereoisometry, *sn*-glycerol 1-phosphate, with isoprenoid groups connected via ether linkages (Pakchung et al., 2006; Peretó et al., 2004; Ulrih et al., 2009). The lipids of *Aeropyrum pernix* K1 are different from those of the anaerobic sulfur-dependent hyperthermophilic archae, in terms of a lack of both tetraether lipids and direct linkages of inositol and sugar moieties (Morii et al., 1999). The isolated polar lipids of *A. pernix* consist solely of 2,3-di-*O*-sesterterpanyl-*sn*-glycerol ($C_{25,25}$-archaeol). Their two major polar lipids are 2,3-di-*O*-sesterterpanyl-*sn*-glycerol-1-phospho-1′-(2′-*O*-α-D-glucosyl)-*myo*-inositol ($C_{25,25}$-archaetidyl(glucosyl)inositol; AGI; about 91 mol%) and 2,3-di-*O*-sesterterpanyl-*sn*-glycerol-1-phospho-*myo*-inositol ($C_{25,25}$-archaetidylinositol; AI; about 9 mol%) (Morii et al., 1999) (Fig. 1). C_{25}-isoprenoid-chain-containing ether lipids were first reported for the haloalkalophiles, including $C_{20,25}$-archaeol and $C_{25,25}$-archaeol (De Rosa et al., 1983; Tindall, 1985). In halophiles, the $C_{25,25}$ molecular species are present in small amounts, and the bulk of the lipids are $C_{20,25}$ and $C_{20,20}$ species. The chain length of the C_{25}-isoprenoid hydrocarbon is 20% longer than those of the C_{20}-isoprenoid and C_{18} straight-chain fatty acids. Therefore, the membranes of the hyperthermophilic archaeon *A. pernix* that are composed of only $C_{25,25}$-archaeol-based lipids are assumed to be 20% thicker than those of the $C_{20,20}$-archaeol-based lipids of other archaea, suggesting that the presence of bipolar tetraether lipids is not necessary for thermal adaptation.

Different physicochemical properties of bipolar tetraether lipid fractions extracted from thermo-acidophilic archaea have been investigated in several studies, including for structure, dynamics, polymorphism, and thermal and mechanical stability (Bartucci et al., 2005; Chong et al., 2005; Khan and Chong, 2000). However, to the best of our knowledge, there has been no previous physicochemical characterization of the $C_{25,25}$ diether polar lipid fractions.

Recently, we investigated the influence of environmental factors (i.e., pH and temperature) on the structural properties of the mem-

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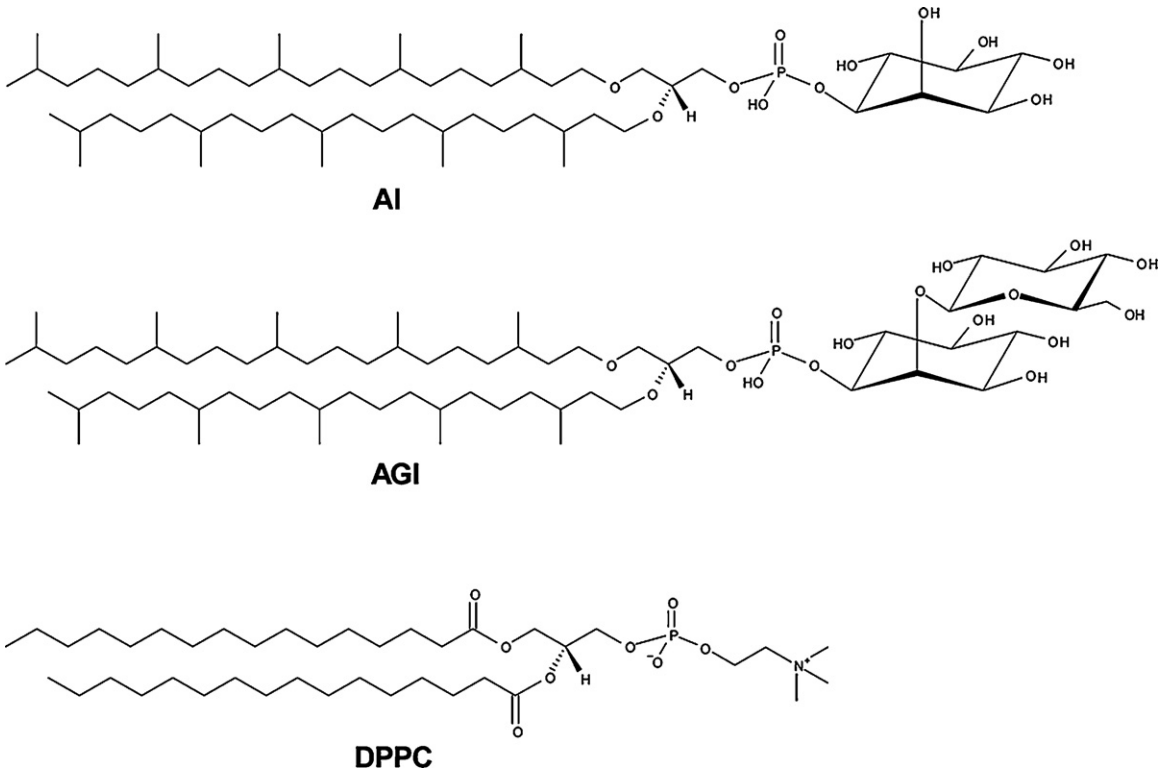

Fig. 1. Structures of 2,3-di-*O*-sesterterpanyl-*sn*-glycerol-1-phospho-*myo*-inositol ($C_{25,25}$-archaetidylinositol) (top: AI), 2,3-di-*O*-sesterterpanyl-*sn*-glycerol-1-phospho-1′-(2′-*O*-α-D-glucosyl)-*myo*-inositol ($C_{25,25}$-archaetidyl(glucosyl)inositol) (middle: AGI) and 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (bottom: DPPC).

brane of *A. pernix in vivo* by electron paramagnetic resonance (EPR), using spin probes and pyrene fluorescence emission spectroscopy (Ulrih et al., 2007). We showed that mean membrane fluidity of *A. pernix in vivo* increases with temperature and depends on pH of the growth medium. Computer simulation of the EPR spectra showed that the membrane of *A. pernix* is heterogeneous and consist of regions that are characterized by three different motional modes, which define three types of membrane domains. The order parameter and the proportion of the spin probes in these three domains define the mean membrane fluidity. The fluidity changes of the membranes with pH and temperature correlated well with the ratio between the fluorescence emission intensity of the first and of the third bands in the vibronic spectra of pyrene, I_1/I_3. Penetration of pyrene into the non-polar membrane region was achieved at temperatures above 65 °C (Ulrih et al., 2007), the lower temperature limit of *A. pernix* growth (Sako et al., 1996).

The membrane of *A. pernix* cells is complex; as well as the lipids; it contains other molecules that can appreciably influence the membrane domain structure. To better understand the structure–function relationships of pure archaeal membrane lipids, we studied the physicochemical properties of bilayer archaeosomes prepared from a polar lipid methanol fraction (PLMF) isolated from *A. pernix* cells grown at 92 °C and pH 7.0. We then investigated the thermal and pH stability of these $C_{25,25}$ archaeosomes by combination of fluorescence emission spectroscopy, differential scanning calorimetry (DSC), and electron paramagnetic resonance (EPR).

2. Materials and methods

2.1. Growth of A. pernix

A. pernix K1 was purchased from the Japan Collection of Microorganisms (number 9820; Wako-shi, Japan). The culture medium (per liter) consisted of 34.0 g synthetic sea salt Reef Salt (Azoo, Taipei, Taiwan), 5.0 g Trypticase Pepton (Becton Dickinson and Company, Sparks, USA), 1.0 g yeast extract (Becton Dickinson and Company, Sparks, USA) and 1.0 g $Na_2S_2O_3 \cdot 5H_2O$ (Sigma–Aldrich, St. Louis, USA). The buffer system used was 20 mM HEPES (Sigma–Aldrich, St. Louis, USA) (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) ($dpK_a/dT = -0.014$) for growth at pH 7.0. The pH of the medium was adjusted to the desired values at 25 °C using 4 M NaOH or HCl, prior to autoclaving. The *A. pernix* cells were grown in 800 mL growth medium inside 1000 mL heavy-walled flasks using a magnetic stirring hot plate and forced aeration (0.5 L min^{-1}) at 92 °C, as described previously (Milek et al., 2005). After 40 h, the suspensions were cooled and centrifuged (Rotanta 460 R, Hettich, Kirchlengern, Germany) in 250 mL Erlenmeyer flasks at 11,000 × *g* for 10 min at 10 °C. The cell pellets were washed twice with the corresponding buffer (20 mM HEPES, pH 7.0, containing 3% NaCl).

2.2. Isolation and purification of lipids, and vesicle preparation

The PLMF was composed of approximately 91% AGI and 9% AI (average molecular weight, 1181.42 g mol^{-1}), and it was purified from lyophilized *A. pernix* cells essentially as described by Bligh and Dyer (1959). The lipids were fractionated with adsorption chromatography on a Sep-Pak® Vac 20 cc Silica cartridges (Waters, Milford, USA) using the following elution sequence: chloroform (170 mL); acetone–methanol (9/1) (250 mL); and methanol (170 mL). The fractions were analyzed by TLC with the solvent of chloroform/methanol/acetic acid/water (85/30/15/5), as described by Morii et al. (1999). This PLMF containing the polar lipids was used for the further analyses. The PLMF was then dried by slow evaporation under a constant flow of dry nitrogen. Following removal of the last traces of organic solvent under a vacuum, the mass of lipids obtained was determined by subtraction (dried finally in a pre-weighed 10-mL round-bottomed glass flask).