Archaea Contain a Novel Diether Phosphoglycolipid with a Polar Head Group Identical to the Conserved Core of Eucaryal Glycosyl Phosphatidylinositol*

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The structure of a major ether polar lipid of the methanogenic archaeon Methanosarcina barkeri was identified as glucosaminyl archaeataidylinositol. This lipid had archaeol (2,3-di-O-phytanyl-sn-glycerol) as a core lipid portion, and the polar head group consisted of 1 mol each of phosphate, myo-inositol and D-GlcN. The polar head group was identified by means of chemical degradations, phosphatidylinositol-specific phospholipase C treatment, permethylation analysis, and fast atom bombardment-mass spectrometry as glucosaminylinositol phosphate, which was linked to the glycerol backbone via a phosophodiester bond. The stereochemical configuration of the phospho-my o-inositol residue of glucosaminyl archaeatidylinositol was determined to be 1-D-myo-inositol 1-phosphate by measuring optical rotation of phospho-my o-inositol prepared by nitrous acid deamination and alkaline hydrolysis from the lipid. 31P NMR of the intact lipid showed that GlcN was linked to C-6 position of myo-inositol as an α-anomer. It is, finally, concluded that the complete structure of this lipid is 2,3-di-O-phytanyl-sn-glycerol-1-phospho-1′(6′-O-(2′-amino-2′-deoxy-α-D-glucopyranosyl))-1′-myo-inositol. This lipid has a hybrid nature of an archaeal feature in alkyl glycerol diether core portion and an eucaryal feature in the polar head group identical to the conserved core structure (GlcNp(α1-6)-myo-inositol 1-phosphate) of glycosylated phosphatidylinositol which serves as a membrane protein anchor in eucaryal cells.

Comparison of small subunit ribosomal RNA base sequences (Woese et al., 1990; Winker and Woese, 1991) and phylogenetic trees of duplicated genes (Iwabe et al., 1989) show that all life on the earth divides into three primary groupings, the Bacteria (formerly euubacteria), the Archaea (formerly archaeabacteria), and the Eucarya (formerly eukaryotes) (Woese et al., 1990; Winker and Woese, 1991). The unique biochemical properties of each primary group support the concept of the three domains (a new rank proposed by Woese et al., 1990). Recent phylogenetic studies have revealed a close relationship of the Archaea to the Bacteria (Iwabe et al., 1989; Woese et al., 1990), and this has been supported by several aspects of biochemical features. The polar lipid of archaea is one of the representative features which distinguishes archaea from the other organisms. The structures of the core lipids (di-O- radyl glycerol portion) of archaeal ether polar lipids are quite different from those of ether lipids of bacteria and eucarya (Langworthy et al., 1982). On the other hand, most of the polar head groups of ether lipids are common to that of ether lipids of the bacteria and eucarya, e.g., phosphoethanolamine, phosphoserine, phospho-my o-inositol, or disaccharides (Nishihara et al., 1989; Nishihara and Koga, 1991). These facts bring up the interest in more detailed analysis of complete structures of ether lipids and in search of a phylogenetic relationship of archaeal and other organisms’ lipids.

Methanosarcina barkeri is an archaeon (archaebacteria) which produces methane from acetate, methanol, or H2 + CO2. Several major polar lipids of M. barkeri were found by thin-layer chromatography (TLC) (Nishihara and Koga, 1991). Four of these structures were determined as hydroxyarchaethidylserine, hydroxyarchaethidyl-myo-inositol (major polar lipids), which have a new ether core lipid (hydroxyarchaeol), and standard arachtaethylserine and arachtaethyl-myo-inositol (minor polar lipids). We have determined the complete structure of the most predominant lipid (designated as PNL6 in Nishihara and Koga (1991)) and report it here. The structure of the polar head group was identical to that of the common part of the eucaryal glycosylated phosphatidylinositol (GPI) membrane anchor while the core lipid was typical of archaeal lipids. This is the first report that shows the apparent relationship in the membrane lipids between the Archaea and the Eucarya.

The nomenclature of archaeal lipids proposed by us (Nishihara et al., 1987) and the expression of absolute stereochemical configurations of phosphoinositol recommended by IUPAC-IUB (1974) are used throughout in this paper.

MATERIALS AND METHODS
Growth of Organism, Extraction and Purification of Lipids—M. barkeri (DSM 800) was grown as reported previously (Nishihara and Koga, 1991). Extraction (Nishihara et al., 1987) and DEAE-cellulose column fractionation of total lipid of M. barkeri were carried out as described previously (Nishihara et al., 1989). PNL6 was eluted from the column by methanol and further purified by TLC with solvent A (see below).

Chromatography—Silica Gel 60 plates (Merck Art 5721) were used for TLC with the following solvents (compositions in volume ratios): solvent A, chloroform/methanol/acetic acid/water (90:30:15:4); solvent B, chloroform/methanol/glycolic acid/water (90:35:15:4); solvent C, chloroform/methanol/concentrated aqueous ammonia (65:35:8); solvent D, chloroform/methanol/acetic acid/water (90:30:15:4); solvent E, light petroleum/diethyl ether/acetic acid (50:50:1). For paper chromatography, solvent F (ethanol/concentrated aqueous ammonia, 3:2, by volume) and Whatman No. 514A paper were used.

Gas-liquid chromatography (GLC) was performed by use of a
Shimadzu GC 9A gas-liquid chromatograph equipped with flame ionization detectors. Hydrocarbons, acetylated or trimethylsilylated derivatives of inositol, were analyzed as previously described (Nishihara et al., 1989). GlcN was chromatographed as an acetylated derivative under the same conditions as those for inositol acetate except that the temperature was 240 °C. Partially methylated alditol acetates were analyzed with a bonded DB-225 column (30 m; film thickness, 0.25 mm; J & W Scientific, USA) with a temperature program of 160–220 °C at 2 °C/min. Chitobiose (GlcN(β1-4)GlcN) was used as the GlcN linkage standard. The amino compound in an aqueous fraction of methanolysate or the hydrolysate of PNL6 was identified and determined by an amino acid analyzer (model 835, Hitachi, Japan).

Analytical Methods and Degradative Procedures—Phosphorus was determined by the method of Bartlett (1959). myo-Inositol and GlcN were estimated by GLC after acetylation with hexacosane as an internal standard. Hydrocarbon chains were prepared from the lipids by hydrolytic acid degradation followed by LiAlH₄ reduction as previously reported (Nishihara et al., 1989). Core lipid of PNL6 was prepared by splitting off the polar head group by acid methanolysis (5% HCl/methanol at 100 °C for 3 h). The polar head group obtained in the aqueous fraction of acid methanolysis of PNL6 was further hydrolyzed by strong acid hydrolysis (6 M HCl, 100 °C, for 18 h). GlcN or GlcNAc of PNL6 was purified and methylated under the same conditions as those for inositol acetate except by paper chromatography with solvent F. The GlcN eluted from the paper was dissolved in 0.085 M HCl for measuring optical rotation. Authentic GlcN HCl salt was used as the standard of optical rotation. Dephosphorylation with HF was performed as described by Morii et al. (1986). Nitrous acid deamination of PNL6 was carried out as follows: To the amino-NH₂ (3–20 mg) dried in a test tube, a 5% m-nitrobenzyl alcohol by using a JMS DX-300 mass spectrometer. Two-dimensional COSY and total correlation spectroscopy (TOCSY) spectra were obtained with a data size of 1024 × 2048 and spectral width of 2500 × 6000 Hz. The mixing time in the latter was 50 ms.

Physical Measurements—IR spectrum was recorded as a thin film using a Shimadzu IR spectrometer IR450S. Optical rotations were measured at 589 nm with a high sensitivity polarimeter (PM-201, Otsuka Electronics, Japan). Fast atom bombardment-mass spectrometry (FAB-MS) was carried out in a positive mode with a matrix of m-nitrobenzyl alcohol by using a JMS DX-300 mass spectrometer (Japan Electron Optics Laboratory, Japan). For 1H NMR, PNL6 was dissolved in chloroform-d/methanol-d₂/water-d₂ (5:104 by volume). Phosphatidylinositol-specific phospholipase C was carried out as described (Taguchi et al., 1989). Permethylylation and preparation of partially methylated alditol acetates were performed by the method of Yang and Hakomori (1971) after acetylation of a free amino group (McConville and Bacc, 1989).

RESULTS

PNL6 was the most predominant polar lipid of M. barleri which comprises 24 mol % of the total polar lipid (Nishihara and Koga, 1991), and it showed positive responses to ninyhdrin, molybdate, and periodate-Schiff reagents and a negative response to the naphthol reagent. The IR spectrum showed absorptions corresponding to groups of methyl-CH₃ and methylene-CH₂– (2840–2950, 1465, and 1375 cm⁻¹), isotropyl–CH(CH₃)₂ (1360 cm⁻¹), ether C–O–C (1110 cm⁻¹), phosphate P=O, P–O–, and P–O–C (1220, 1100, and 1050 cm⁻¹), amino–NH₂ (3390, 1640, and 1560 cm⁻¹) and hydroxy OH (3400 cm⁻¹).

Structure of the Core Lipid—GLC of hydrocarbon chain prepared from PNL6 showed only one peak which coincided with phytane. A chloroform-soluble product of acid methanolyis of PNL6 was cochromatographed with archaeol by TLC with solvent E. The specific optical rotation, [α]D, of the product was +8.50°, which was identical to that (+8.43°) of authentic archaeol. This result confirmed that the core lipid of PNL6 had the stereochemical configuration of 2,3-di-O-phytanyl-sn-glycerol which was common to archaeal polar lipids.

Structure of the Polar Head Group—Acid methanolyis completely cleaved the polar head group, and all of the phosphorus was recovered as organic phosphate in the methanol/water phase after the Bligh and Dyer partition (1959). Analyses of the methanol/water-soluble product by GLC (after acetylation) under the conditions described under “Materials and Methods” and by an amino acid analyzer showed no peak on the chromatograms. When the product was further hydrolyzed with 6 M HCl at 100 °C for 18 h, free GlcN was detected by an amino acid analyzer, and myo-inositol and GlcN were found by GLC after acetylation or trimethylsilylation. The molar ratio of inorganic phosphate, myo-inositol, and GlcN in these products was 1:1:0.95:0.86. The optical rotation [α]D of GlcN from PNL6 was +71°, which was identical to that of D-GlcN (+73°). FAB-MS of the intact PNL6 gave the molecular ion peak of m/z 1056 (M + 1)⁺. FAB-MS of methanolysate of PNL6 revealed a peak m/z 422 (M + 1)⁺ corresponding to the molecular weight (M = 421) of glucosaminyl-myo-inositol phosphate and an accompanying peak m/z 436 (M' + 1)⁺ corresponding to the monomethylated form (M' = 435), respectively. These results confirmed that the structure of PNL6 is glucosaminyl archaeolinositol (archaeol + phosphate + myo-inositol + D-GlcN, molecular weight = 1055).

The fact that the acid methanolyis completely degraded PNL6 to yield archaeol suggested that archaeol and inositol were linked via phosphodiester linkage because the glycosyl bond of the glucosamine was resistant to acid hydrolysis due to the presence of the NH₂ group. To determine the sequence of the constituents of the polar head group, the following analysis by chemical reactions was carried out. Dephosphorylation with HF yielded archaeol as the major chloroform-soluble product accompanied with a small amount of archaeaic acid (phosphonomoester of archaeol). The chloroform-soluble product of NaN02 treatment gave a single major spot on TLC that comigrated with authentic archaeolinositol on TLC with solvents A, B, or C. The positive ion FAB-MS spectrum of the product showed the molecular ion peak of m/z 917 (M + Na⁺) which was consistent with the molecular weight of archaeolinositol (M = 894). Phosphatidylinositol-specific phospholipase C treatment of intact PNL6 yielded archaeol as a chloroform-soluble product. N-Acetylated PNL6 showed mobilities relative to PNL6 of 1.55 and 0.80 on TLC with solvents A and D, respectively. Partially methylated alditol acetate of GlcNAc prepared from PNL6 cochromatographed with GLC on 2-N-methyl acetamido-3,4,6-tri-O-methyl-1,5-di-O-acetyl-2-deoxyglucitol prepared from N-acetylated chitobiose. This indicated that the GlcN is the pyranose form and present as the terminal end of the polar head group linking at the C-1 position. These results established that D-GlcN was directly linked to myo-inositol to which archaeol was linked via a phosphodiester linkage. Inositol phosphate was obtained from archaeolinositol prepared from PNL6 by removal of GlcN with NaN02. The
specific optical rotation ([α]D) of the resultant inositol 1-phosphate was −9.9°, which coincided to 1-O-1-phospho-myoinositol (−9.8°) of soybean phosphatidylinositol (Pizer and Ballou, 1959; Ballou and Pizer, 1960).

1H NMR of PNL6 was carried out to determine the configuration of GlcN and position of the inositol moiety at which GlcN was linked. When the spectrum was recorded as the solution of chloroform-d6/methanol-d3/water-d2, all the resonances from the polar head group were clearly separated and assigned (Fig. 1) by decoupling and 2D-DQFCOSY and TOCSY spectra. The coupling pattern of H-1 of the inositol residue confirmed that the phosphate group is linking to the C-1 position. The chemical shift of the anomeric proton of GlcN (5.57 ppm, ΔJ1,2 = 3.8 Hz) indicates that it is linked as an α-isomer. Since the linking position of GlcN is C-1, H-1 of GlcN was irradiated to examine the nuclear Overhauser effect (NOE). However, interresidual NOE was scarcely observed in this solvent (data not shown). On the other hand, a strong NOE was observed between H-1 of GlcN and H-6 of the inositol residue (Fig. 2A), while the spectrum was not so clearly resolved (Fig. 2B) when the spectrum was recorded in a solvent of chloroform-d6/methanol-d6. The difference in the strength of NOE in two solutions may be ascribed to the difference in conformations of the inositol-GlcN moiety in these solvents. The NOE observed shows that H-1 of GlcN is close to H-6 of the inositol residue. From the model building of the glucosaminyl-myo-inositol moiety, the linkage of C-1 of GlcN to either C-1, C-5, or C-6 of the inositol residue may be consistent with the NOE observation. The possibility of the linkage to H-1 of inositol was, however, excluded because of the coupling between H-1 of inositol and phosphorus (Fig. 1) and the optical rotation of the phospho-myo-inositol indicating the presence of phosphate at the C-1 position. Then, the 1H NMR spectrum of archaetidylinositol-myo-inositol prepared from PNL6 by the NaN02 deamination was recorded and compared with that of PNL6 (Table I). The difference in the chemical shifts was largest at H-1 and H-6. Consequently, it can be concluded that C-1 of GlcN was linked to the C-6 position of myo-inositol. Finally it is concluded that the complete structure of PNL6 is 2,3-di-O-phytanyl-sn-glycerol-1-phospho-1'-[6'-O-(2"-amino-2"-deoxy-α-D-glucopyranosyl)]-1''-D-myo-inositol (glucosaminyl archaetidyl-myo-inositol) (Fig. 3).

DISCUSSION

The present study establishes the structure of glucosaminyl archaetidylinositol of M. barkeri. This is a novel lipid which consists of archaeol and glucosaminyl-(phospho)-myo-inositol. The core portion, archaeol (2,3-di-O-phytanyl-sn-glycerol) is typical of archaeal polar lipids. However, the structure of the polar head group is noteworthy. Glucosaminyl inositol is distinctively unique because of its identical structure to the polar head group of glucosaminyl phosphatidylinositol in eucarya. The position of myo-inositol at which GlcN is bound and the anomeric configuration (GlcN(α1-6)inositol) of M.

![Fig. 1. 1H NMR spectrum of glucosaminyl archaetidylinositol in chloroform-d6/methanol-d6/water-d2 (5:10:4 by volume).](image)

![Fig. 2. 1H NMR spectra of glucosaminyl archaetidylinositol in chloroform-d6/methanol-d6 (2:1 by volume). A, one-dimensional spectrum of nuclear Overhauser effect spectroscopy (NOESY). B, one-dimensional spectrum. Resonances in B were identified by 2D-DQFCOSY and TOCSY (data not shown). Tetramethylsilane was used as an internal standard (0 ppm). The spectrum of the aliphatic region was omitted. G, I, gro, and -C-1- mean protons of GlcN, inositol, glycerol, and C-1 carbon of phytanyl chains, respectively.](image)

![Fig. 3. Structure of glucosaminyl archaetidylinositol. Numbers of the inositol ring represent carbon numbering of 1-d-myo-inositol.](image)
Glucosaminyl Archaetidylinositol

barkeri glucosaminyl archaetidylinositol are completely identical to the corresponding part of eucaryal GPI membrane anchors. This structure is a characteristic one found as the conserved structure only in eucaryal GPI membrane anchors (Ferguson and Williams, 1988; Thomas et al., 1990). Glucosaminyl phosphatidylinositol is formed during biosynthesis of GPI (Doering et al., 1989, 1990; Masterson et al., 1989). Glucosaminyl archaetidylinositol is, therefore, a diether analogue of glucosaminyl phosphatidylinositol, and it may be assumed that this lipid is a “hybrid lipid” that has archaean nature in the core portion and a eucaryal feature in the polar head group. Glucosaminyl archaetidylinositol is the first archaean polar lipid, to our knowledge, that shares the characteristic with eucarya-specific lipids. The only glycoconjugate found in Archaea is a polymannose- and glycerol ether lipid-containing lipopolysaccharide from Thermoplasma acidophilum (Mayberry-Carson et al., 1974), which does, however, not have an eucaryal characteristic.

Despite the wide variety of glycan structures of GPI membrane anchors found in various eucarya, the GlcN(α1-6)-1-d-myo-inositol 1-phosphate structure is conserved in all the anchors so far analyzed, showing that the structure is the basic one for the anchors. Because of the identical structure of the conserved head group, it is likely that they are synthesized via similar pathways. Studies on biosynthesis and its enzymology would provide us important information as to a phylogenetic relationship of the Archaea and the Eucarya in lipid biochemistry. Genetic analysis of the responsible enzymes may help a comparative study.

The numerous proteins that are bound to a membrane by a GPI anchor play important roles in cell physiology, and free GPI has been implicated as a source of second messengers in response to insulin (Saltiel et al., 1986; Chan et al., 1989). The role of glucosaminyl archaetidylinositol in this archaeon is not known at this stage.

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