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Heptads of Polar Ether Lipids of an Archaebacterium, *Methanobacterium thermoautotrophicum*: Structure and Biosynthetic Relationship†

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ABSTRACT: The structures of the eight major polar lipids of *Methanobacterium thermoautotrophicum* were determined. They were one diether glycolipid (gentiobiosylarchaeol) and serine-, inositol-, and ethanolamine-containing diether and tetraether types of phospholipids and phosphoglycolipids [archaetidyl-L-serine, caldarchaetidyl-L-serine, gentiobiosylcaldarchaetidyl-L-serine, 1D-1-(archaetidyl)-*myo*-inositol, 1D-1-(caldarchaetidyl)-*myo*-inositol, 1D-1-(gentiobiosylcaldarchaetidyl)-*myo*-inositol, archaetidylethanolamine]. In combination with 2 neutral lipids and 3 polar lipids that have been already described in the previous paper [Nishihara, M., Morii, H., & Koga, Y. (1987) *J. Biochem. (Tokyo) 101*, 1007], the 13 lipids were proposed to be classified in three groups, that is, three "heptads", each of which was constituted by diether and tetraether types of neutral lipids, glycolipids, and phospholipids and a tetraether phosphoglycolipid. The heptad concept implied the biosynthetic relationship between diether and tetraether lipids which was supported by in vivo kinetic experiments. When growing cells were pulse labeled with [^{32}P]orthophosphate, there was a lag of 15–90 min between the rapid incorporation of label into diether polar lipids and that of label into the corresponding tetraether polar lipids. The lag times and rates of incorporation of ^{32}P into tetraether phospholipids and their respective diglucosyl derivatives (phosphoglycolipids) were almost identical. In a pulse–chase experiment with [^{32}P]P_i, rapid turnover of the three diether lipids other than archaetidylethanolamine was observed. At the same time radioactivity was incorporated into gentiobiosylcaldarchaetidylinositol and other tetraether polar lipids. These results are consistent with a model which postulates that head-to-head condensation of phytanyl chains of two diether polar lipids occurs to yield tetraether polar lipids.

One of the prominent distinguishing features of archaebacteria is the presence of glycerol isopranyl ether lipids. The complex lipids are classified as diether and tetraether types of lipids by their hydrophobic core portion. Because the tetraether types of lipids are apparently made of two halves of diether types of lipids in structure, it is important to elucidate the structural and biosynthetic relationship of the two types of lipids in one archaebacterium. For this purpose, structure determination of a whole set of major polar lipids in a methanogen is desirable, because, in contrast with extreme halophiles and sulfur-dependent archaebacteria, methanogens have both types of lipids (Balch et al., 1979; Tornabene & Langworthy 1979). Although several polar lipids have been reported in various methanogenic bacteria, only one report on the structure analysis of the major polar lipids in one species (*Methanospirillum hungatei*; Kushuwaha et al., 1981) has appeared.

Recently, the lipid composition and structures of three tetraether polar lipids of *Methanobacterium thermoautotrophicum* were reported (Nishihara & Koga, 1987; Nishihara et al., 1987). These lipids were caldarchaetidylethanolamine, gentiobiosylcaldarchaeol, and gentiobiosylcaldarchaetidylethanolamine. We have proposed in that paper the concept of "a quartet of lipids" which consisted of the three lipids along with bare caldarchaeol. Moreover, it has been suggested that a quartet would be extended to a heptad by the addition of three diether lipids. Kramer et al. (1987) have found archaetidylethanolamine, which should be a diether component of the heptad, in *M. thermoautotrophicum*. As previously reported (Nishihara & Koga, 1987), this organism has at least 23 species of polar lipids, only 4 of which were structurally elucidated as described above. In this paper the structures of eight more polar diether and tetraether lipids of *M. thermoautotrophicum* are determined, and three sets of complete heptads of serine, inositol, and ethanolamine lipids are described.

Although the heptad concept is conceived on a structural basis, it implies the biosynthetic relationship of diether and tetraether types of polar lipids. Therefore, the hypothetical relationships should be experimentally examined. Up to now, mechanisms of biosynthesis of tetraether lipid from diether lipids in archaebacteria remain speculative. On the basis of the examination of the structural regularities in the tetraether lipids, Langworthy (1985) and De Rosa and Gambacorta (1986) inferred that the condensation of diether neutral lipid occurred before attachment of polar head groups to the diether

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lipid, while Langworthy (1985) did circumspectly not exclude the possibility that the condensation could occur via a diether lipid already substituted with polar head groups. On the other hand, Kushwaha et al. (1981) discussed, on the basis of the structures of seven polar diether and tetraether lipids of *M. hungatei*, that biosynthesis of tetraether polar lipids might occur by head-to-head condensation of diether polar lipids. Therefore, a primary problem, at present, is whether the head-to-head condensation of alkyl groups occurs between two molecules of free archaeols or between diether lipids which have been already substituted by polar head groups. It is expected that kinetic studies of the heptads with radioactive phosphate may discriminate the two possibilities of the mechanisms. The biosynthetic relationship of diether and tetraether polar lipids may give fundamental support to the heptad concept. The present paper also describes that the incorporation kinetics supports the possibility of condensation of diether polar lipids. Temporary designations of polar lipids of *M. thermoautotrophicum* shown in Figure 2 of our previous paper (Nishihara & Koga, 1987), the nomenclature of archaebacterial lipids proposed by us (Nishihara et al., 1987),[1] and the expression of absolute stereochemical configurations of inositol recommended by IUPAC–IUB (1974) are used in this text.

Experimental Procedures

Growth Conditions. M. thermoautotrophicum ΔH (DSM 1053) was grown semicontinuously in a 10-L glass carboy as described previously (Nishihara & Koga, 1987). The cells were harvested by centrifugation.

Extraction and Purification of Lipids. Total lipid of the bacterial cells was extracted with a trichloroacetic acid acidified solvent by the method of Bligh and Dyer (1959) as modified by Nishihara and Koga (1987). Total lipids (700–800 mg) were roughly fractionated on a column (2.5 × 40 cm) of (diethylaminoethyl)cellulose (Merck, acetate form) by elutions with 2 L each of I (chloroform), II (methanol), III (acetic acid), and IV (0.25 M ammonium acetate containing methanol). Glycolipids (GL1a and GL1b) and ethanolamine lipids (PNL1a, PNL1b, and PNGL1) were eluted in fraction II. Serine lipids (PNL2a, PNL2b, and PGL2) and some unidentified ninhydrin-positive lipids were found in fraction III. Inositol lipids (PL2a, PL2b, and PGL1) and unidentified phospholipids appeared in fraction IV. Each lipid from the fractions was purified by thin-layer chromatography (TLC)[2] with solvent B (glycolipids and ethanolamine lipids) or solvent C (serine lipids and inositol lipids).

Chromatography. Silica gel 60 plates (Merck Art 5721) were used for TLC with the following solvents (compositions in volume ratio): solvent A, chloroform–methanol–7 M aqueous ammonia (65:35:8); solvent B, chloroform–methanol–acetic acid–water–acetone (200:150:15:10:100); solvent C, chloroform–methanol–acetic acid–water (85:30:15:5); solvent D, light petroleum–ethyl ether–acetic acid (50:50:1). Solvents A and C were used for two-dimensional TLC in the first and second dimensions, respectively. Spots were visualized on silica gel TLC plates by using ninhydrin, acid molybdate, α-naphthol, periodate–Schiff reagent, and 30% H_2SO_4 as described (Nishihara & Koga, 1987). Cellulose TLC was developed with either solvent E, ethanol–1.4 M aqueous ammonia (3:2), for glycerophosphoserine (GPS) or solvent F, phenol–water (100:38), for glycerophosphoinositol (GPI). Spots on the cellulose TLC plates and chromatography papers were detected with salicylsulfonic acid/$FeCl_3$ reagent (phosphoric esters; Vorbeck & Marinetti, 1965) and ninhydrin.

Gas–liquid chromatography (GLC) was performed by use of a Shimadzu GC 9A gas–liquid chromatograph equipped with flame ionization detectors. Hydrocarbons were analyzed on a column (2 m) of 2% Dexsil 300GC on Chromosorb W AW/DMCS (80–100 mesh) at a temperature increasing from 100 to 330 °C at a rate of 10 °C/min. Acetylated inositol and trimethylsilylated inositol (TMS-inositol) were chromatographed on a 3-m glass column packed with 2% OV-225 on Uniport HP (60–80 mesh) at a temperature increasing from 160 to 220 °C at a rate of 2 °C/min (acetylated inositol) or at a constant temperature of 165 °C (TMS-inositol).

Analytical Methods and Degradative Procedures. Phosphorus, sugar, glycerol, and protein were determined by the methods of Bartlett (1959), Dubois et al. (1956), Lambert and Neish (1950), and Lowry (1951), respectively. *myo*-Inositol was estimated on GLC after acetylation with acetylated mannitol as an internal standard.

Acid methanolysis, acetolysis (Nishihara & Koga, 1987), and mild alkaline methanolysis (Kates, 1972) were performed as described. The chloroform-soluble products were analyzed on TLC with solvent B for glycolipids, solvent C for phospholipids, and solvent D for neutral lipids.

Hydrocarbon was prepared by HI cleavage of the ether linkages followed by $LiAlH_4$ reduction as described by Kates et al. (1965) with modifications as follows. Lipid was suspended in 3 mL of 55% HI and heated at 100 °C for 1 h. After extraction of the resultant alkyl iodide with light petroleum, it was reduced to the corresponding alkane with 50 mg of $LiAlH_4$ in 2 mL of ethyl ether–chloroform (9:1 by volume) for 1 h at 100 °C.

Dealkylation with BCl_3 was carried out to prepare glycerophosphoric esters by the method of Nishihara and Koga (1988) with minor modifications for serine lipids in which Bligh and Dyer partitioning of the cleavage products was omitted in order to minimize unexpected degradation of GPS. GPI and GPS were analyzed by cellulose TLC.

Preparation of alditol acetates, acetylated methyl glucosides, or partially methylated alditol acetates and CrO_3 oxidation of glycolipids were as previously reported (Nishihara et al., 1987).

Serine was liberated from ether serine lipids (PNL2a, PNL2b, and PGL2) and authentic diacylphosphatidylserine by HF hydrolysis (Morii et al., 1986) and purified by paper chromatography on Toyo No. 514A paper with solvent G [1-butanol–acetic acid–water (3:3:1)]. After conversion of the purified serine to *N,O*-bis(3,5-dinitrobenzoyl) derivatives, the optical isomers were analyzed by high-performance liquid chromatography (HPLC) as described (Oi et al., 1983; Morii & Koga, 1986).

The position of phosphoric ester on the inositol residue in inositol lipids (PL2a, PL2b, and PGL1) was determined essentially as described by Pizer and Ballou (1959). Inositol lipids were hydrolyzed in 2 M KOH at 100 °C for 1 h. After

[1] The terms of archaebacterial lipids proposed by Nishihara et al. (1987) and used in this paper are defined as follows: archaeol, 2,3-di-*O*-alkyl-*sn*-glycerol diether; caldarchaeol, 2,2′,3,3′-di-*O*-biphytanediyl-bis(*sn*-glycerol) tetraether; archaetidyl-X and caldarchaetidyl-X, archaeol and caldarchaeol linked with phospho-X by a phosphodiester linkage (X is a water-soluble alcohol residue such as serine, ethanolamine, or inositol), that is, diether and tetraether analogues of phosphatidyl-X, respectively.

[2] Abbreviations: GLC, gas–liquid chromatography; HPLC, high-performance liquid chromatography; TLC, thin-layer chromatography; FAB-MS, fast atom bombardment mass spectrum; GPS, glycerophosphoserine; GPI, glycerophosphoinositol; TMS, trimethylsilyl; I-1-P, *myo*-inositol 1-monophosphate; I-2-P, *myo*-inositol 2-monophosphate.

neutralization of water-soluble products with Dowex AG-50X4 resin, it was applied to paper chromatography developed with solvent H [2-propanol–14 M aqueous ammonia–water (7:1:2)]. Authentic *myo*-inositol 1-monophosphate (I-1-P) and *myo*-inositol 2-monophosphate (I-2-P) were run in parallel as mobility standards. The major phosphorus-containing products were I-1-P and I-2-P. Because the two inositol monophosphates were developed very closely on the paper especially when a preparative amount was developed, the bands corresponding to I-1-P and I-2-P were eluted in one fraction with water. After estimation of optical rotation of the inositol phosphate mixture, a small amount of the mixture was separated by paper chromatography into I-1-P and I-2-P. The ratio of I-1-P and I-2-P was measured by phosphorus determination. Specific rotation of I-1-P was calculated on the basis of the fact that I-2-P was not optically active.

Physical Measurements. Optical rotations were measured at 25 °C at 589 nm with a high-sensitivity polarimeter (PM-71, Union Scientific Engineering Co., Japan). Fast atom bombardment mass spectra (FAB-MS) were obtained with a mass spectrometer (JMS DX-300, Japan Electron Optics Laboratory, Japan), with glycerol plus 15-crown-5 as a matrix in a negative ion mode (Fujii et al., 1985).

Incorporation of ^{32}P. For ^{32}P-labeling experiments, medium 2 (Balch et al., 1979) was modified as the phosphate concentration was reduced to 0.4 mM and MOPS [3-(*N*-morpholino)propanesulfonic acid] was added to give a final concentration of 50 mM (pH 7.0). The organism was grown successively twice at 65 °C in 10 mL of the low-phosphate medium containing [^{33}P]P_i (5 Ci/mol of P_i) under a pressurized atmosphere of H_2 + CO_2 + H_2S (78:22:0.2) in a 500-mL shaking flask for 24 h while shaking. Finally, 70 mL of the same medium containing the same specific radioactivity of [^{33}P]P_i was inoculated with 7 mL of the last subculture. After the addition of [^{32}P]P_i (10 Ci/mol of Pi) at the early log phase of growth, 6–12 mL of sample was taken at the times indicated up to 18 h. An aliquot (3–8 mL) of the sample received 4 mg of unlabeled carrier cells and was chilled. The cells were collected by centrifugation and stored at −20 °C until lipid extraction. Protein was determined in the other aliquot (3–4 mL) of the sample after precipitated by 5% trichloroacetic acid. Protein concentration of the culture represented the bacterial growth. Labeled lipids were extracted from cells suspended in 0.8 mL of water as described above. Individual lipid species (even diether and tetraether lipids with the same polar group) were clearly separated on two-dimensional TLC. The ratio of $^{32}P/^{33}P$ represents specific radioactivity of ^{32}P because labeling with [^{33}P]P_i was long enough to equilibrate with cellular phosphate.

Pulse–Chase Experiments. At early to midlog phase of growth in 55 mL of the low-phosphate medium, [^{32}P]P_i was added (18.2 Ci/mol of P_i) and the culture was allowed to continue to grow. After 90 min of pulse labeling, anoxic 1 M potassium phosphate (pH 7.0) solution was added to the culture to give the final concentration of 35 mM phosphate (88 times higher than that of pulse labeling culture). Samples were taken and processed as in the incorporation experiments.

Counting of Labeled Lipids. Spots were detected by autoradiography. Radioactive lipids adsorbed to silica gel were suspended in 5.5 mL of liquid scintillator [Scintisol EX-H–water (10:1)]. Radioactivity was measured with a liquid scintillation spectrometer (Packard 2660) 3 days after suspension to complete elution of lipids from Merck silica gel 60 powder. Complete elution was essential to avoid an uncorrectable shift of the β-ray spectrum of ^{32}P to the lower energy channel. Radioactivities of double-labeled samples were corrected for fractional spillovers.

Table I: General Characterization of Lipids from *M. thermoautotrophicum*

	GL1b	PNL2a	PNL2b	PGL2	PL2a	PL2b	PGL1
staining on TLC							
molybdate	−	+	+	+	+	+	+
α-naphthol	+	−	−	+	−	−	+
ninhydrin	−	+	+	+	−	−	−
IO_4^-–Schiff	+	−	−	+	+	+	+
P:sugar:glycerol	0:2:1	1:0:2	1:0:1	1:2:2	1:0:2	1:0:1	1:2:2
core lipid[a]	A	C	A	C	C	A	C
hydrocarbon[b]	C_{20}	C_{40}	C_{20}	C_{40}	C_{40}	C_{20}	C_{40}
GP ester[c]		GPS	GPS	GPS	GPI	GPI	GPI
sugar chain[d]	Gen			Gen			Gen
FAB-MS[e]	975	1466	818	1790	1541	893	1865

[a] The glycerol ether core portion of lipids was prepared by acid methanolysis from the glycolipids and the inositol lipids and by acetolysis and subsequent acid methanolysis from the serine lipids. A and C represent archaeol and caldarchaeol, respectively. [b] C_{20} and C_{40} are phytane and biphytane, respectively. [c] Glycerophosphoric ester. [d] Gen shows a gentiobiosyl chain. [e] Mass of $[M - 1]^-$ ion is shown.

Materials. Authentic samples of archaeol and phytane were prepared from archaetidylglycerol purified from the total lipids of *Halobacterium cutirubrum* which was a gift from Dr. Kamekura. Caldarchaeol and biphytane were prepared from gentiobiosylcaldarchaeol of *M. thermoautotrophicum* lipids. L-Serine, D-serine, and *myo*-inositol were purchased from Tokyo Kasei Chemicals, Japan. 1D-1-Phospho-*myo*-inositol and 2-phospho-*myo*-inositol were obtained from Sigma. GPS and GPI were prepared by mild alkaline methanolysis from phosphatidylserine (Sigma, bovine brain) and phosphatidylinositol (Sigma, soybean), respectively. [^{32}P]P_i and [^{33}P]P_i were purchased from New England Nuclear. Scintisol EX-H was the product of Dojindo Laboratories, Kumamoto, Japan.

Results

The stereochemical configurations of both glycerol diether and tetraether cores of total lipids from *M. thermoautotrophicum* were previously described as *sn*-2,3-substituted glycerol (Nishihara et al., 1987).

Glycolipids (GL1a and GL1b). GL1a, one of two glycolipids, has been identified as gentiobiosylcaldarchaeol [β-D-glucopyranosyl-(1→6)-β-D-glucopyranosyldibiphytanediyl-bis(glycerol) tetraether; Figure 1d] (Nishihara et al., 1987). The another one, GL1b, was characterized as summarized in Table I. The sugar was identified as glucose on GLC chromatograms after conversion to acetylated methyl glycoside and to alditol acetate. These results indicate that GL1b is a diphytanylglycerol diether having a glucosylglucose moiety attached to the glycerol. GLC analysis showed that the partially methylated alditol acetates derived from permethylated GL1b coincided with 1,5-diacetyl-2,3,4,6-tetramethylglucitol and 1,5,6-triacetyl-2,3,4-trimethylglucitol prepared from isomaltose. These were present in a nearly equimolar ratio. CrO_3 oxidation of the acetylated GL1b completely destroyed the glucose in the lipid. Both glucosyl residues were, therefore, found to have β-configuration since CrO_3 preferentially oxidizes β-anomers of hexosides (Laine & Renkonen, 1975). The glucose in the lipid molecule of this organism has already been determined to be the D enantiomer (Nishihara et al., 1987). Finally, the structure of GL1b was identified as gentiobiosylarchaeol [β-D-glucopyranosyl-(1→6)-β-D-glucopyranosyldiphytanylglycerol; molecular weight = 976; Figure 1c].

Serine Lipids (PNL2a, PNL2b, and PGL2). Several characteristics of these lipids are shown in Table I. Acid methanolysis caused no change in these lipids except for PGL2

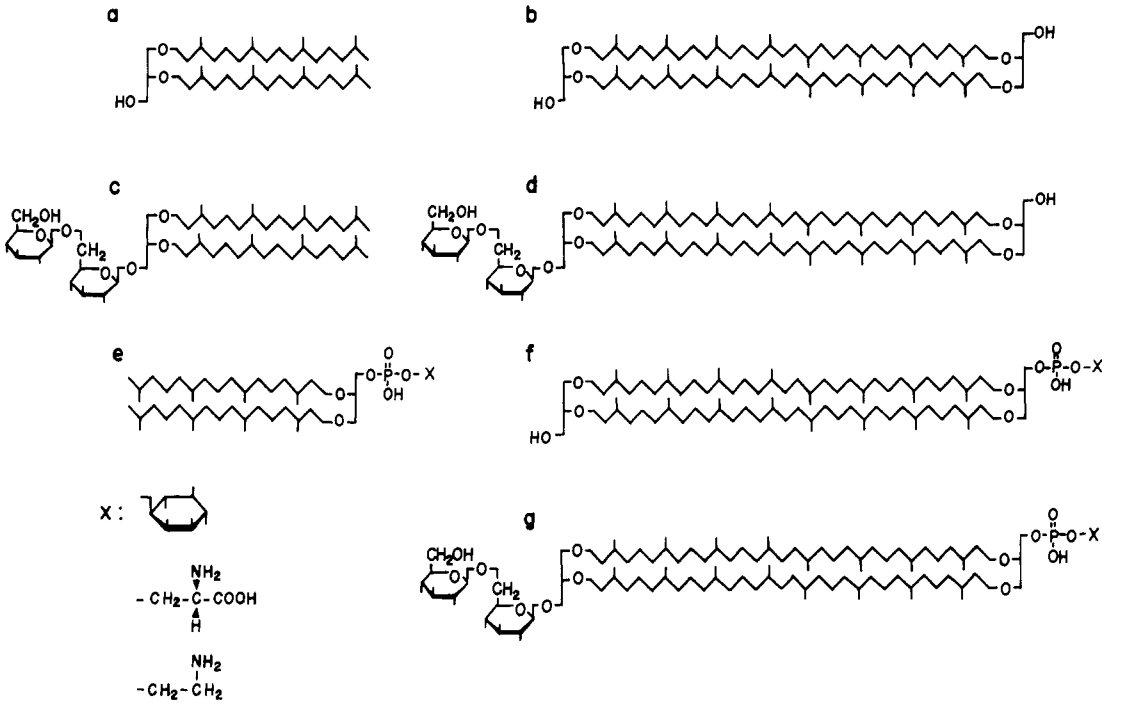

FIGURE 1: Structure of heptad lipids of *M. thermoautotrophicum*: (a) archaeol; (b) caldarchaeol; (c) gentiobiosylarchaeol; (d) gentiobiosylcaldarchaeol; (e) archaetidyl-X; (f) caldarchaetidyl-X; (g) gentiobiosylcaldarchaetidyl-X. X is either serine, inositol, or ethanolamine.

(see below). GPS was recovered almost quantitatively from each of the three lipids upon BCl_3 treatment. On the basis of these results, the structure of PNL2a is concluded to be caldarchaetidylserine [dibiphytanediylbis(glycerol) tetraether with phosphoserine; molecular weight = 1467; Figure 1f, X = serine]. Significant peaks other than $[M - 1]^-$ were m/z 1467 ($[M]^-$), 1379 ($[M - CH_2CHNH_3COO]^-$), and 1378 ($[M - CH_2CHNH_3COO - 1]^-$) in negative ion FAB-MS.

The results depicted in Table I show that PNL2b is diphytanylglycerol diether having phosphoserine as a polar head group. This lipid completely coincided on two-dimensional TLC with archaetidylserine from *Methanobrevibacter arboriphilus* A2 (Morii et al., 1986; Koga et al., 1987). Thus, PNL2b was identified as archaetidylserine (diphytanylglycerophosphoserine; molecular weight = 819; Figure 1e, X = serine).

Acid methanolysis of PGL2 released the sugar moiety to yield a phospholipid which comigrated with PNL2a on TLC. The water-soluble product was identified as glucose by GLC of acetylated methyl glycoside or alditol acetate. Acetolysis and subsequent mild alkaline methanolysis yielded a glycolipid which had the same R_f value as gentiobiosylcaldarchaeol on TLC. Permethylation analysis of the glucoside chain of PGL2 on GLC and CrO_3 oxidation showed the same results as those of GL1b. The significant peaks other than $[M - 1]^-$ in FAB-MS were m/z 1791 ($[M]^-$) and 1702 ($[M - CH_2CHNH_3COO - 1]$). These results and the data shown in Table I indicate that PGL2 has the structure of dibiphytanediylbis(glycerol) tetraether having phosphoserine at one glycerol residue and a gentiobiosyl group at another glycerol residue. That is, PGL2 was gentiobiosylcaldarchaetidylserine [β-D-glucopyranosyl-(1→6)-β-D-glucopyranosyldibiphytanediylbis(glycerol) tetraether with phosphoserine; molecular weight = 1791; Figure 1g, X = serine].

Serine moieties were obtained from these serine lipids and authentic diacylphosphatidyl-L-serine by HF hydrolysis. The HPLC analysis of the *N,O*-bis(3,5-dinitrobenzoyl) derivative of the resultant serine showed that the serine residues of all the lipids were the L enantiomers (Table II).

Table II: Stereochemical Analysis of Dinitrobenzoyl Derivatives of Serine Prepared from Serine Lipids by HPLC

source of serine	percentage[a] of L isomer	percentage[a] of D isomer
diacylphosphatidyl-L-serine	96.2	3.8
caldarchaetidylserine (PNL2a)	98.0	2.0
archaetidylserine (PNL2b)	98.6	1.4
gentiobiosylcaldarchaetidylserine (PGL2)	>99.0	<1.0[b]
authentic D-serine	5.8	94.2
authentic L-serine	98.9	1.1

[a] The values were calculated from the peak areas of the HPLC chromatogram of dinitrobenzoyl derivatives of serine. [b] Peak area of the D isomer was smaller than the lower limit (1.0% of L isomer) of the integration.

Inositol Lipids (PL2a, PL2b, and PGL1). General characterization of each of these lipids is summarized in Table I. Dealkylation of the lipids with BCl_3 yielded GPI quantitatively. Free inositol was obtained by strong acid hydrolysis of GPI and proved to be *myo*-inositol on GLC of acetylated and TMS derivatives. These data indicate that PL2a is a dibiphytanediylbis(glycerol) tetraether with phosphoinositol attached to one of the glycerol moieties. PL2a was, therefore, identified as caldarchaetidyl-*myo*-inositol [dibiphytanediylbis(glycerol) tetraether with phospho-*myo*-inositol; molecular weight = 1542; Figure 1f, X = *myo*-inositol].

PL2b was identified from the data shown in Table I as a diphytanylglycerol diether with phosphoinositol attached to the glycerol, that is, archaetidyl-*myo*-inositol (diphytanylglycerophospho-*myo*-inositol; molecular weight = 894; Figure 1e, X = *myo*-inositol).

PGL1 was an inositol-containing phosphoglycolipid. The molar ratio of phosphorus to inositol was 1:1.1. Strong alkaline hydrolysis or consecutive treatments of acetolysis and mild alkaline methanolysis yielded GL1a which was identified by mobilities and responses to the spray reagents on TLC and GLC identification of sugar. These results combined with those indicated in Table I suggest that PGL1 was a dibiphytanediylbis(glycerol) tetraether with two polar head groups of glucosylglucose and phospho-*myo*-inositol separately atta-

Table III: Optical Rotations of I-1-P Obtained by Strong Alkaline Hydrolysis from the Inositol Lipids

source of phosphoinositol	$[\alpha]_D$ of I-1-P: free form	$[\alpha]_D$ of I-1-P: dicyclohexylamine salt
caldarchaetidylinositol (PL2a)	−9.5	+3.4
archaetidylinositol (PL2b)	−9.9	+3.8
gentiobiosylcaldarchaetidylinositol (PGL1)	−9.2	+4.2
soybean phosphatidylinositol	ND[a]	+3.9
soybean phosphatidylinositol (1D-1-phospho-*myo*-inositol)	(−9.8)[b]	(+3.4)

[a] Not determined. [b] Values in parentheses are data from Pizer and Ballou (1959).

ched on glycerol moieties. In negative ion FAB-MS, significant peaks other than $[M - 1]^-$ were recorded at m/z 1866 ($[M]^-$), 1703 ($[M - C_6H_{11}O_5]^-$), 1702 ($[M - C_6H_{11}O_5 - 1]^-$), 1541 ($[M - C_{12}H_{21}O_{10}]^-$), and 1540 ($[M - C_{12}H_{21}O_{10} - 1]^-$). Permethylation analysis of the glucoside chain by GLC and CrO_3 oxidation gave the same results as those of GL1b. Finally, the structure of PGL1 was concluded to be gentiobiosylcaldarchaetidyl-*myo*-inositol [β-D-glucopyranosyl-(1→6)-β-D-glucopyranosyldibiphytanediylbis(glycerol) tetraether with phospho-*myo*-inositol; molecular weight = 1866; Figure 1g, X = *myo*-inositol].

Structures of phosphoinositol moieties of the three inositol lipids were determined by the method of Pizer and Ballou (1959) with minor modification. The strong alkaline hydrolysis yielded a mixture of I-1-P and I-2-P from each inositol lipid. All the inositol lipids were readily hydrolyzed in dilute acid. If the phosphatidyl group was esterified at position 4, 5, or 6 of inositol, mild acid treatment would cause no change on the lipid because hydroxyl groups at the positions were all-trans in conformation so that the cyclic phosphate intermediate could not be formed. Specific rotations of the I-1-P in free acid and salt forms coincided to the values of authentic 1D-1-phospho-*myo*-inositol as shown in Table III. This result indicates that the phosphoric residue is linked to the 1-position of the inositol residue in all three lipids and the 1-phospho-*myo*-inositol is the D enantiomer.

Ethanolamine Lipids. The structures of PNL1a and PNGL1 have been identified as caldarchaetidylethanolamine (Figure 1f, X = ethanolamine) and gentiobiosylcaldarchaetidylethanolamine (Figure 1g, X = ethanolamine) in the previous paper (Nishihara et al., 1987). We have independently identified PNL2b as archaetidylethanolamine (Figure 1e, X = ethanolamine). The data are not presented here because the structure of this lipid in this bacterium has already been reported by Kramer et al. (1987).

Kinetics of Incorporation of $[^{32}P]P_i$ into Phospholipids. In order to examine the biosynthetic relationship between diether and tetraether types of phospholipids, pulse-labeling experiments were carried out to study the kinetics of incorporation of inorganic phosphate into diether and tetraether phospholipids and phosphoglycolipids. The ratio $^{32}P/^{33}P$ representing ^{32}P specific activity in total lipid increased linearly with time for about 15 h after a lag period of 20–30 min. After 15 h, the curve was almost flat (data not shown). The initial lag reflects the slow labeling of several of the major lipids. During these 18 h of labeling, protein content of the culture increased exponentially 4-fold, and 2% of $[^{32}P]P_i$ in the medium was incorporated into total lipid. These indicate that cells were normally growing and $[^{32}P]P_i$ was rapidly taken up by the cells. The kinetics of $[^{32}P]P_i$ incorporation into the individual phospholipids of *M. thermoautotrophicum* are shown in Figure 2. It can be seen that ^{32}P labeling of three diether phospholipids, archaetidylserine, archaetidylinositol, and archaetidylethanolamine, occurred with little or very short lag, indicating rapid equilibration of precursor pools, but ^{32}P labeling of the corresponding tetraether phospholipids, caldarchaetidylserine, caldarchaetidylinositol, and caldarchaetidylethanolamine, occurred after a lag of 15–90 min, and the initial rates were lower than those of diether counterparts. It can also be seen that labeling of the phosphoglycolipids, gentiobiosylcaldarchaetidylethanolamine, gentiobiosylcaldarchaetidylinositol, and gentiobiosylcaldarchaetidylserine, became linear after a lag of 60–90 min. The lag periods and rates of increase of specific radioactivity in the bipolar phosphoglycolipids were identical within experimental errors with those of the tetraether phospholipids containing the corresponding phosphoric ester head groups. These results suggest that the phosphoric ester portions of tetraether phospholipids passed through a larger pool of precursors.

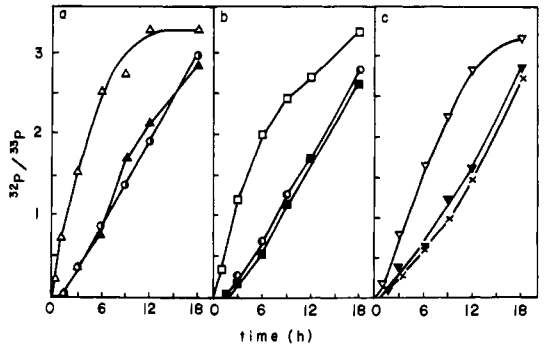

FIGURE 2: Incorporation of ^{32}P into *M. thermoautotrophicum* lipids. Cells were continuously labeled with $[^{33}P]P_i$ and then pulsed with $[^{32}P]P_i$ in the presence of $[^{33}P]P_i$. At the times indicated, samples were removed, and lipids were extracted and separated by two-dimensional TLC. $^{32}P/^{33}P$ ratio represents ^{32}P specific radioactivity. (a) (△) Archaetidylserine; (▲) caldarchaetidylserine; (◑) gentiobiosyl caldarchaetidylserine. (b) (□) Archaetidylinositol; (■) caldarchaetidylinositol; (◐) gentiobiosylcaldarchaetidylinositol. (c) (▽) Archaetidylethanolamine; (▼) caldarchaetidylethanolamine; (×) gentiobiosylcaldarchaetidylethanolamine.

Turnover of Phospholipids during a Chase Period following Pulse Labeling with $[^{32}P]P_i$. Pulse–chase experiments, in which $[^{32}P]P_i$ was incorporated during a 90-min pulse and was chased by the addition of a high concentration of unlabeled potassium phosphate (pH 7.0), were carried out to detect turnover of phospholipids. The protein concentration of the culture increased 1.7-fold during the first 200-min chase period without a lag time. Rapid turnover was observed in archaetidylinositol, archaetidylserine, and a diether phospholipid (PL5) (Figure 3). (PL5 was tentatively identified as archaetidic acid from its mobility and staining responses on TLC and the presence of archaeol and glycerol phosphate as its components.) That is, the radioactivities (not specific radioactivities) of these lipids decreased rapidly with nearly the same rate after an initial rise during the first 10 min of the chase period. Archaetidylethanolamine was the only diether phospholipid that incorporated label for about 100 min after the chase. The changes in the relative ^{32}P specific radioactivities of archaetidylserine and archaetidylethanolamine (calculated from ^{32}P radioactivity in each lipid and content of cell protein which was proportional to phospholipid content) suggest an apparent precursor–product relationship between archaetidylserine and archaetidylethanolamine (data not shown); that is, archaetidylethanolamine appeared to be derived directly from archaetidylserine. This is consistent with the fact that ^{32}P incorporation into archaetidylethanolamine showed a rather

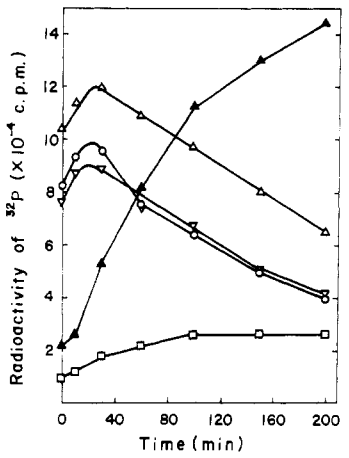

FIGURE 3: Chase of ^{32}P in phospholipids and phosphoglycolipids of *M. thermoautotrophicum* after pulse labeling. Culture grown in the low-phosphate medium (55 mL) received [^{32}P]P_i, and incubation was continued. After 90 min, 2 mL of anoxic 1 M potassium phosphate (pH 7.0) was added, and incubation was continued. Samples were removed at the times indicated and were processed as in the incorporation experiment. Radioactivity of each lipid extracted from cells in 4.5-mL portions of the culture was plotted. (○) Archaetidylserine; (▽) archaetidylinositol; (△) "archaetidic acid"; (□) archaetidylethanolamine; (▲) gentiobiosylcaldarchaetidylinositol.

Table IV: Balance Sheet of ^{32}P Radioactivity in Lipids between 30 and 150 min after Chase[a]

lipid	difference of ^{32}P (cpm)[b]
gentiobiosylcaldarchaetidylinositol	+77 368
caldarchaetidylinositol	+2 655
gentiobiosylcaldarchaetidylserine	+5 438
caldarchaetidylserine	+184
gentiobiosylcaldarchaetidylethanolamine	+1 149
caldarchaetidylethanolamine	<+30
archaetidylethanolamine	+7 340
loss from lipids	+23 480
sum of increase	+117 644
archaetidylinositol	−38 865
archaetidylserine	−45 905
PL5 ("archaetidic acid")[c]	−37 634
sum of decrease	−122 404

[a] Experimental procedures are shown in the legend of Figure 2. [b] Difference in ^{32}P radioactivity between lipids taken at 150 min and 30 min after chase. [c] PL5 is an archaetidic acid like phospholipid but not yet finally identified.

longer lag time compared with the other diether phospholipids (Figure 2).

On the other hand, the tetraether phospholipids and phosphoglycolipids incorporated more or less ^{32}P during the chase period (Table IV). The sum of the decrease in radioactivities of diether phospholipids was balanced by the sum of the increase in ^{32}P of tetraether phospholipids and others. Although the most significant increase in radioactivity was observed in gentiobiosylcaldarchaetidylinositol (Figure 3), its specific radioactivity was kept at a low level during the period because the content of the lipid was the largest (Nishihara & Koga, 1987).

Discussion

The complete structures of the major polar lipids of *M. thermoautotrophicum* were established in this paper. Three of these lipids (caldarchaetidylserine, gentiobiosylcaldarchaetidylserine, and gentiobiosylcaldarchaetidylinositol) were novel ether lipids. Two of the others (archaetidylinositol and caldarchaetidylinositol) were found for the first time in methanogenic bacteria while they had been found in sulfur-dependent archaebacteria [*Thermococcus celar* (De Rosa et al., 1987); *Sulfolobus solfataricus* (De Rosa et al., 1986)]. Gentiobiosylarchaeol and archaetidylserine have already been described in methanogens [*Methanococcus voltae* (Ferrante et al., 1986); *Methanobrevibacter arboriphilus* (Morii et al., 1986)], and now they are found also in *M. thermoautotrophicum* as described here. This is the first determination of the complete structure of archaebacterial inositol lipids including stereochemistry and the position on the inositol where the phosphoric group is esterified.

In addition to these lipids, four lipids (gentiobiosylcaldarchaeol, caldarchaetidylethanolamine, gentiobiosylcaldarchaetidylethanolamine, and archaetidylethanolamine) are present in *M. thermoautotrophicum* (Nishihara et al., 1987; Kramer et al., 1987). These 11 species of polar lipids contain only four kinds of polar head groups. Of these, phospholipid and phosphoglycolipid are easily classified into three groups on the basis of phosphoric ester polar head groups (phosphoserine, -ethanolamine, and -inositol). Each of the three groups of lipids consists of a diether form of phospholipid and tetraether forms of phospholipid and phosphoglycolipid. It appears that the tetraether lipids are structurally composed of two molecules of diether lipids, that is, archaeol, gentiobiosylarchaeol, and archaetidyl-X (X = ethanolamine, serine, or inositol). Therefore, one group of lipid can be expanded to contain four tetraether lipids (phospholipid, glycolipid, phosphoglycolipid, and neutral lipid) and three diether lipids which correspond to the structural halves of the tetraether lipids. Archaeol and caldarchaeol were identified in the lipid extract of this organism (Nishihara & Koga, 1987). It is, thus, proposed here that the seven lipids are united in "a heptad of lipids" (Figure 1). A heptad is characterized by a phosphoric ester head group. Thus, the major lipids of *M. thermoautotrophicum* were grouped into three (ethanolamine, serine, and inositol) heptads. Because two glycolipids and two neutral lipids are common to all heptads, three heptads contain 13 lipids, calculated as

$$(7 - 4) \times 3 + 4 = 13$$

On the basis of structure analysis of 13 lipids of *M. thermoautotrophicum*, the heptad concept is characterized by three rules for the combination of polar head groups on the core lipids as follows: (1) The same kind of polar head group found in diether lipids is present also in tetraether lipids and vice versa. (2) One polar head group (in this case, sugar chain, serine, and phospho-*myo*-inositol) found in tetraether lipids has the same stereochemical structure as that of the corresponding diether lipid. (3) When polar head groups are bound separately to two glycerol residues of tetraether lipids, these are not the same. Besides, one is a glycosyl residue, and the other is a phosphoric ester. The three rules can be easily perceived from the structures of the *M. thermoautotrophicum* lipids. The last one was also supported by the fact that there has been in other reports no example of tetraether lipid in archaebacteria which has two glycosyl residues or two phosphoric ester polar head groups in one molecule at both ends (Kushwaha et al., 1981; De Rosa et al., 1986; Lanzotti et al., 1987).

Not only structural regularity but biosynthetic relationship between diether and tetraether lipids was suggested to be possibly included in the heptad concept on the basis of in vivo kinetic experiments as discussed below. On examination of each heptad of polar lipids of *M. thermoautotrophicum*, one is tempted to speculate that the biosynthesis of tetraether polar lipids might occur by head-to-head condensation of diether

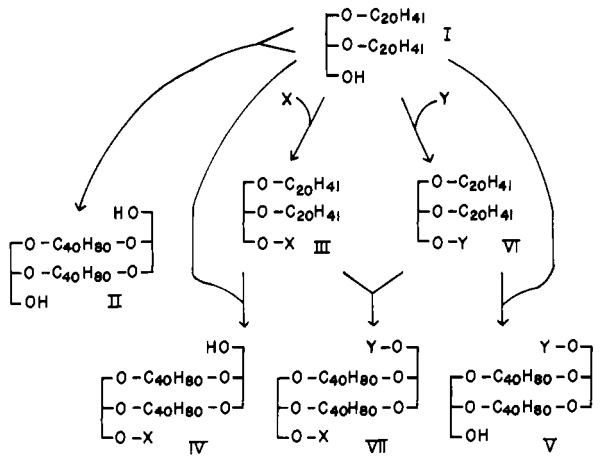

FIGURE 4: A proposed pathway of biosynthesis of tetraether polar lipids. $-C_{20}H_{41}$ and $-C_{40}H_{80}-$ represent phytanyl and biphytanediyl groups, respectively. (X) Water-soluble phosphoric ester moiety; (Y) sugar residue. (I) Archaeol; (II) caldarchaeol; (III) diether phospholipids, e.g., archaetidylserine, archaetidylinositol, and archaetidylethanolamine; (IV) tetraether phospholipids, e.g., caldarchaetidylserine, caldarchaetidylinositol, and caldarchaetidylethanolamine; (V) gentiobiosylcaldarchaeol; (VI) gentiobiosylarchaeol; (VII) tetraether phosphoglycolipids, e.g., gentiobiosylcaldarchaetidylserine, gentiobiosylcaldarchaetidylinositol, and gentiobiosylcaldarchaetidylethanolamine.

polar lipids. This possible pathway postulates that the condensation between molecules of the diether type of phospholipids, glycolipids, or archaeol yields the tetraether type of phospholipids, glycolipids, or phosphoglycolipids, or free caldarchaeol (Figure 4). On the basis of this pathway, it is expected that the incorporation of $[^{32}P]P_i$ into diether phospholipids precedes the incorporation into tetraether types of phospholipids and phosphoglycolipids. That is, $[^{32}P]P_i$ must be incorporated into tetraether phospholipids via the corresponding diether phospholipid. Because the pool sizes of diether phospholipids in *M. Thermoautotrophicum* are not so small, the lag time of ^{32}P labeling of tetraether phospholipids would be appreciably longer than those of diether lipids. The kinetics of ^{32}P incorporation into phospholipids shown in Figure 2 is consistent with this prediction but not with an alternative pathway in which two molecules of archaeol are condensed to caldarchaeol which is then converted to tetraether polar lipids by attachment of polar head groups. The incorporation experiments also showed that tetraether phospholipid and phosphoglycolipid in the same heptad were labeled with an identical lag time and with an identical rate. This result is consistent with the prediction from the pathway shown in Figure 4.

The rapid turnover of two diether phospholipids, archaetidylinositol and archaetidylserine, is another line of evidence for the pathway of Figure 4. This phenomenon indicates that diether polar lipids of this organism are metabolic intermediates which also support the proposed pathway as above. The diether polar lipids are not end products. An apparently rapid turnover of the small amount of [^{14}C]mevalonate-labeled diether polar lipids in *Thermoplasma* (Langworthy, 1982) is consistent with our model. Although turnover products of three diether phospholipids could not be identified, the total loss of ^{32}P from diether lipids could be almost accounted for by the incorporation of ^{32}P during the chase period into the tetraether type of phospholipids. This is consistent with the model shown in Figure 4. Because another phospholipid (PL5) which underwent rapid turnover has not yet been finally identified, that should not be included at present in the lines of evidence for the pathway. Archaetidylethanolamine is an exception of diether lipid turnover. This is probably explained as follows. The pulse–chase experiment suggested that archaetidylserine was the precursor of archaetidylethanolamine. In the experiment shown in Figure 3, conversion of pulse-labeled archaetidylserine to archaetidylethanolamine and turnover of the latter lipid would have simultaneously occurred, and the former phenomenon would be mainly observed because ^{32}P equilibration with cellular phosphate had not been established (it was a pulse-labeling and chase experiment). Therefore, this is not considered to be evidence against the pathway shown in Figure 4.

We have described three rules of the heptad concept as above. The first two rules are obviously logical consequences from our model of tetraether lipid biosynthesis. Therefore, the heptad hypothesis not only is based on the structural regularity but also includes the mechanism of tetraether lipid biosynthesis. The last one, lack of a tetraether lipid which possesses two glycosyl residues or two phosphoric ester groups, cannot be accounted for by knowledge obtained to date. Further studies on the biosynthesis of tetraether lipids in biomembranes are necessary to explain the last rule.

Heptad-like groups of polar lipids have also been described in other archaebacteria. For example, the seven polar lipids along with archaeol and caldarchaeol found in *M. hungatei* (Kushuwaha et al., 1981) can be considered as two incomplete heptads. "Incomplete" means that caldarchaetidylglycerol has not been found. Kushwaha et al. (1981) and Ferrante et al. (1987), who have revised a lipid structure of this organism, also suggested the condensation of diether polar lipids. In *S. solfataricus*, five phospho- and phosphoglycolipids are considered to be members of two "quartets of polar lipids". "Quartet" is defined as the tetraether members of heptads as described in Nishihara et al. (1987) for ethanolamine-containing tetraether lipids. In *M. hungatei* and *Sulfolobus*, their heptads or quartets are characterized by two kinds of glycosyl groups but not by a common phosphoric ester group in contrast with *M. thermoautotrophicum*. *M. arboriphilus* was found to contain a complete inositol–gentiobiose heptad (Morii et al., 1988) which was identical with that of *M. thermoautotrophicum*, reflecting that both organisms belong to the same family. Thus, complete or incomplete heptads or quartets of polar lipids are widely found in archaebacteria, and there is a possibility that the heptad hypothesis could be a general rule of archaebacterial lipids, while further structural and biosynthetic investigations are required to establish it.

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End-Label Fingerprintings Show That an N-Terminal Segment of Depactin Participates in Interaction with Actin†

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ABSTRACT: A 1:1 complex of actin and depactin, an actin-depolymerizing protein isolated from starfish oocytes [Mabuchi, I. (1983) *J. Cell Biol. 97*, 1612-1621], was cross-linked with 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide (EDC) to introduce covalent bonds at their contact site. Locations of cross-linking sites were identified along the depactin sequence by the end-label fingerprinting, which employed site-directed antibodies against the N- and C-termini of depactin as end labels. Mappings with these end labels have revealed that the N-terminal segment of depactin (residues 1-20) contains sites in contact with the N- and C-terminal segments of actin, both of which participate in interaction with depactin [Sutoh, K., & Mabuchi, I. (1986) *Biochemistry 25*, 6186-6192].

Actin is one of major components of cytoskeletal structures and motile apparatus in eukaryotic cells. Association and dissociation of actin and a large number of actin-binding proteins are essential for a dynamic change of cytoskeletons and motile apparatus in living cells. In order to understand the molecular mechanism underlying these events, it is important to elucidate the structural basis of interaction between actin and actin-binding proteins.

Since there are many types of actin-binding proteins in a cell while surface area on actin, especially on F-actin, is very limited, it is a reasonable speculation that some actin-binding proteins share their binding sites with others. In fact, it has been shown that the N- and C-terminal segments of actin participate in binding many actin-binding proteins: myosin (Sutoh, 1982a), depactin (Sutoh & Mabuchi, 1984, 1986), fragmin (Sutoh & Hatano, 1986), troponin I (Grabarek & Gergely, 1987), α-actinin, actinogelin (Mimura & Asano, 1987), gelsolin (K. Sutoh and H. L. Yin, unpublished results), and profilin (T. Pollard, D. Kaiser, C. Ampe, and J. Vandekerckhove, personal communication). In spite of the wide

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