In Vitro Biosynthesis of Ether-Type Glycolipids in the Methanoarchaeon

*Methanothermobacterthermautotrophicus*

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The biosynthesis of archaean ether-type glycolipids was investigated in vitro using *Methanothermobacterthermautotrophicus* cell-free homogenates. The sole sugar moiety of glycolipids and phosphoglycolipids of the organism is the β-D-glucosyl-(1→6)-D-glucosyl (gentiobiosyl) unit. The enzyme activities of archaeol:UDP-glucose β-glucosyltransferase (monoglucosylarchaeol [MGA] synthase) and MGA:UDP-glucose β-1,6-glucosyltransferase (diglucosylarchaeol [DGA] synthase) were found in the methanoarchaeon. The synthesis of DGA is probably a two-step glucosylation: (i) archaeol + UDP-glucose → MGA + UDP, and (ii) MGA + UDP-glucose → DGA + UDP. Both enzymes required the addition of K⁺ ions and archaeatidylinositol for their activities. DGA synthase was stimulated by 10 mM MgCl₂, in contrast to MGA synthase, which did not require Mg²⁺. It was likely that the activities of MGA synthesis and DGA synthesis were carried out by different proteins because of the Mg²⁺ requirement and their cellular localization. MGA synthase and DGA synthase can be distinguished in cell extracts greatly enriched for each activity by demonstrating the differing Mg²⁺ requirements of each enzyme. MGA synthase preferred a lipid substrate with the sn-2,3 stereostructure of the glycerol backbone on which two saturated isoprenoid chains are bound at the sn-2 and sn-3 positions. A lipid substrate with unsaturated isoprenoid chains or sn-1,2-dialkylglycerol configuration exhibited low activity. Tetraether-type caldarchaetidylinositol was also actively glucosylated by the homogenates to form monoglucosyl caldarchaetidylinositol and a small amount of diglucosyl caldarchaetidylinositol. The addition of Mg⁴⁺ increased the formation of diglucosyl caldarchaetidylinositol. This suggested that the same enzyme set synthesized the sole sugar moiety of diether-type glycolipids and tetraether-type phosphoglycolipids.

More than 100 novel archaean membrane lipid structures have been elucidated in the past 40 years (6, 14, 17, 18). These are unusual in the stereoconfiguration of the glycerol backbone, ether bonds, isoprenoid chains, and bipolar tetratereth structures, which are one of the most remarkable features of archa (17).

In vitro studies of the major pathway of polar lipid biosynthesis in archaean have been published over these past 15 years and extensively reviewed (16). Nishihara and Koga discovered sn-glycero-1-phosphate formation activity from dihydroxyacetone phosphate in a cell-free homogenate of *Methanothe

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thesis of the specific archaeal lipid structures. One of the questions is which is the real substrate, unsaturated archaeol or saturated archaeol. The biosynthetic intermediates of archaeal phospholipids are ether compounds with unsaturated geric-keranyl chains. The second question is whether this enzyme phospholipids are ether compounds with unsaturated gera-
thesis of the specific archaeal lipid structures. One of the ques-}

"chaeol (DGA) from archaeol via 

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is specific to archaeal lipids with

nylgeranyl chains. The second question is whether this enzyme

phospholipids are ether compounds with unsaturated gera-
thesis of the specific archaeal lipid structures. One of the ques-

/H11002

The pelleted cell paste was stored at

20°C until use.

/J9252

was washed with

20°C until use.

M. thermautotrophicus

/H9262

as an enzyme source unless specified otherwise, the lipid substrate was replaced

with 10 mg of the enzyme was added 24 h after

the incubation started. Archaeoldehydinolinositol, DGA, and diglucoyl caldarchaeti-
dinylositol (DCCI) were purified from the total lipid of

M. thermautotrophicus

by TLC using solvent B (see below). Monoglucosylcaldarchaeol and 1,1'-digu-
clusylcaldarchaeol (caldarchaeol with a glucose unit on each hydroxyl group) were

chemically synthesized from caldarchaeol in place of archaeol in the chemical

synthesis reaction of MGA (see above). Negative FAB-MS of the products

displayed m/z 1462 (M–H)– and m/z 1624 (M–H)–, respectively, which

were consistent with the molecular weights of monoglucosylcaldarchaeol and 1,1-
'diglucoylcaldarchaeol.

Enzyme preparation. Frozen cells (about 9 g [wet weight]) of

M. thermautotrophicus

were suspended in 10 ml of buffer A or buffer B (see above) containing 1 mg

of DNaS I (Sigma) and were passed through a French pressure cell operated at

1,400 kg/cm². This process was repeated three times. Cell debris and unbroken

cells were removed by 10 min of centrifugation (10,000 × g). Each homogenate

was centrifuged at 100,000 × g for 2 h to separate supernatant and membrane fractions. The membrane fraction was washed once and resuspended with the same

buffer. The supernatant, membrane, and washing fractions prepared in buffer A were designated fraction AS, fraction AM, and fraction AW, respectively. The supernatant, membrane, and washing fractions prepared in buffer B were designated fraction BS, fraction BM, and fraction BW, respectively.

Enzyme assay. (ii) MGA synthase. The complete assay mixture (final volume,

0.1 ml) contained the supernatant fraction of

M. thermautotrophicus

cell homogenate (fraction AS, 440 µg of protein) unless otherwise indicated, 0.1 M Bicine buffer (pH 8.0) DojinLaboratories, Kumamoto, Japan, 0.5 M KCl, 20 nmol archaeoldehydinolinositol, 20 nmol archaeol, and 100 nmol UDP-[U-14C]glucose (18.5 Bq/nmol; Perkin-Elmer Life Sciences). Archaeol and archaeoldehydinolinositol were dispersed with the aqueous components of the reaction mixture, except for EDTA, DNaS I, and the enzyme preparation, in a 1.5-ml microtubete and the reaction was incubated at 40°C for 30 min in a BRANSONIC 1210 (BRANSON) bath. After the addition of UDP-glucose and enzyme preparation, the reaction mixture was incubated in the 1.5-ml microtube at 60°C for 60 min. The reaction was stopped by adding 1 ml of 0.1 M HCl in methanol, and the reaction mixture was transferred to a 10-ml screw-cap glass tube with 1.5 ml of 0.1 M HCl in methanol and 2.5 ml CHCl₃. Finally, 2.15 ml of 1 M MgCl₂ (pH 2) was added to the mixture to partition the reactants and the products into aqueous and organic layers. After being mixed twice with 0.1 M HCl/methanol-1 M MgCl₂ (pH 2) (1:0.8 [vol/vol]), the organic layer was evaporated to dryness and examined for radioactivity.

(ii) DGA synthase. The assay for DGA synthase was similar to that for MGA synthase except that the membrane fraction of the

M. thermautotrophicus

cell homogenate (fraction AM or fraction BM, 160 µg of protein) was usually used as an enzyme source unless specified otherwise, the lipid substrate was replaced by 40 nmol MGA, and 10 mM MgCl₂ was added. TLC TLC was carried out on a Silica Gel 60 plate (Merek) with the following solvents: solvent A, which consisted of chloroform, methanol, and 7 M ammonia (60:35:8); and solvent B, which consisted of chloroform, methanol, acetic acid, and water (80:30:15:5). Lipid spots were detected by spraying acid molybdate reagent (7) for phospholipids and subsequent charring for all lipids. Radioactive spots on a TLC plate were recorded with a Fujifilm FLA-2000 fluor image analyzer with an imaging plate (Fujifilm type BAS-MS).

Identification of reaction products. To confirm the structural identity of the enzyme reaction products from archaeol, MGA, or caldarchaeol, the reaction mixture was scaled up 20 to 400 times using nonradioactive UDP-glucose instead of the radiolabeled UDP-glucose. The complete assay mixture (final volume, 1 ml) contained the supernatant fraction of

M. thermautotrophicus

cell homogenate (fraction AS, 440 µg of protein) unless otherwise indicated, 0.1 M Bicine buffer (pH 8.0), 20 nmol archaeoldehydinolinositol, 20 nmol archaeol, 100 nmol UDP-[U-14C]glucose (18.5 Bq/nmol; Perkin-Elmer Life Sciences). Archaeol and archaeoldehydinolinositol were dispersed with the aqueous components of the reaction mixture, except for EDTA, DNaS I, and the enzyme preparation, in a 1.5-ml microtubete and the reaction was incubated at 40°C for 30 min in a BRANSONIC 1210 (BRANSON) bath. After the addition of UDP-glucose and enzyme preparation, the reaction mixture was incubated in the 1.5-ml microtube at 60°C for 60 min. The reaction was stopped by adding 1 ml of 0.1 M HCl in methanol, and the reaction mixture was transferred to a 10-ml screw-cap glass tube with 1.5 ml of 0.1 M HCl in methanol and 2.5 ml CHCl₃. Finally, 2.15 ml of 1 M MgCl₂ (pH 2) was added to the mixture to partition the reactants and the products into aqueous and organic layers. After being mixed twice with 0.1 M HCl/methanol-1 M MgCl₂ (pH 2) (1:0.8 [vol/vol]), the organic layer was evaporated to dryness and examined for radioactivity.

Identification of reaction products. To confirm the structural identity of the enzyme reaction products from archaeol, MGA, or caldarchaeol, the reaction mixture was scaled up 20 to 400 times using nonradioactive UDP-glucose instead of UDP-[U-14C]glucose, and the incubation time was prolonged (3 h). Either the products (MGA and DGA) were purified by TLC with solvent A or monoglu-
cosyl caldarchaetidylinositol (MGCI) and DGCI were purified with solvent B,

"FIG. 1. Possible pathways of β-D-glucosyl-(1→6)-β-D-glucosyl ar-

chaeol (DGA) from archaeol via β-D-glucosyl archaeol (MGA).

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and the products were analyzed by FAB-MS. Sugar moieties in MGA and DGA were analyzed by NMR spectrometry.

Analytical methods and physical measurements. Phosphate (2) and protein (34) were determined as described previously. A FAB-MS spectrum was recorded with a mass spectrometer (JEOL JMS DX-303) with a matrix of glycerol plus 15-crown-5 in a negative mode or with a matrix of glycerol in a positive mode. 'H-NMR spectra (500 MHz) were recorded on a Bruker DRX-500 spectrometer in CDCl3 for MGA and CDCl3-CD3OD (7:3) for DGA. The optical rotation was measured at room temperature at 589 nm with an automatic digital polarimeter (PM-201; Photob, Japan). Radioactivity was counted using a liquid scintillation spectrometer (LSC-3500E; Aloka, Japan) with Aquasol-2 (Packard) as the scintillator.

RESULTS

Activity of glucosylarchaeol synthesis. Because bacterial glyceroglycolipids are synthesized from diacylglycerol by sequential glycosylation with nucleotide-linked sugars, analogous substrates were tested for detection of in vitro glycolipid-synthesizing activity in M. thermautotrophicus cell homogenates. When archaeol was incubated with radioactive UDP-[14C]glucose in the presence of cell-free homogenates of M. thermautotrophicus under the conditions described in Materials and Methods, 14C was incorporated into chloroform-soluble materials. The incorporation was dependent on the incubation time and protein concentration of the added cell homogenates (0 to 660 μg) (data not shown) and continued for at least 60 min at 60°C (Fig. 2A). The addition of archaeolipidinositol was essential for the activities (see below for details). The time courses of MGA and DGA synthesis from archaeol and of DGA synthesis from MGA are shown in Fig. 2A and C, respectively. Twenty to 45% of each lipid substrate was converted to the products during the reaction. To confirm that the chloroform-soluble products were really archaeol-derived glycolipids, they were analyzed by TLC with solvent A and FAB-MS. Two spots were detected by TLC after the reaction from archaeol (MGA synthase reaction) (Fig. 2B). One radioactive spot comigrating with standard MGA (Rf = 0.80) was the main product. The other minor spot (Rf = 0.50) coincided chromatographically with standard DGA. Only one spot was detected in the reaction from MGA (DGA synthase reaction) (Fig. 2D) and showed the same mobility in TLC as standard DGA. The lipid products enzymatically prepared from archaeol or MGA were purified by TLC with solvent A and confirmed by identification of their molecular ions. The FAB-MS of the major lipid product and the other minor lipid product from archaeol gave signals of m/z 814 (M-H)− (Fig. 3A) and m/z 976 (M-H)−, respectively, which are consistent with the molecular weights of MGA and DGA, respectively. FAB-MS of the product from MGA gave signals of m/z 976 (M-H)− and m/z 814 (M-glucose)− (Fig. 3B). The 'H-NMR spectra of MGA and DGA clearly showed that all glucoside linkages had the β configuration (MGA: δ 4.36, d, J = 8.0 Hz; DGA: δ 4.33, d, J = 7.8 Hz, δ 4.28, d, J = 7.8 Hz) (Fig. 4). Further, the 'H-NMR spectrum of the enzymatically prepared DGA was identical to that of the synthetic β-D-glucosyl-(1→6)-β-D-glucosyl archaeol (1). These results show that M. thermautotrophicus cell homogenates contain activities that synthesize DGA via MGA from archaeol. These enzymes are MGA synthase (archaeol:UDP-glucose β-glucosyltransferase) and DGA synthase (MGA:UDP-glucose β-1,6-glucosyltransferase).

FIG. 2. Time courses of MGA and DGA synthesis from archaeol (A and B) and DGA synthesis from MGA (C and D). (A and B) Synthesis of glycolipids from 100 nmol UDP-[U-14C]glucose and 20 nmol archaeol catalyzed by the supernatant fraction of M. thermautotrophicus homogenate fraction AS incubated in the presence of 0.5 M KCl and 20 nmol archaeolipidinositol in a final volume of 0.1 ml at 60°C, pH 8.0. (C and D) The enzyme assay condition was similar to that of the above assay except that the membrane fraction of M. thermautotrophicus homogenate fraction BM was used as an enzyme source, the lipid substrate was replaced by 40 nmol MGA, and 10 mM MgCl2 was added. (B and D) Autoradiograms of one-dimensional TLC of 14C products taken at indicated times (shown in minutes).

Cellular localization of the activities. Because many polar lipid-synthesizing enzymes are associated with membranes, the cellular localization of the glycolipid-synthesizing enzymes was examined. Table 1 shows the distribution of the MGA synthase and DGA synthase activities between the particulate (membrane) and soluble fractions of the cell homogenates prepared at pH 7.5 and 6.5. The synthesis activities of the two glycolipids were found in both the soluble and membrane fractions. Almost all the MGA synthase activity was recovered in the soluble fraction when the homogenates were prepared at pH 7.5 (Table 1, fraction AS), while a greater proportion of DGA synthase was found to be associated with the membrane fraction (Table 1, fraction AM). The specific activities showed that MGA synthase was concentrated in the soluble fraction (Table 1, fraction AS), with DGA synthase in the membrane fraction (Table 1, fraction AM). The distribution of the activities between the two fractions (soluble and membrane) varied depending on the pH under which the cell homogenates had been prepared. When prepared at pH 6.5, more activities were recovered in the membrane fraction. The membrane fraction...
prepared at pH 6.5 (Table 1, fraction BM) contained the largest amount and highest specific activity of DGA synthase. Because fraction AS contained almost all of the MGA synthase activity, it was usually used as the source of MGA synthase (unless otherwise indicated). Fraction BM was usually used as the source of DGA synthase. Although these fractions contained other enzyme activity along with the major activity, the two enzyme activities could be distinguished by their different requirements for Mg$^{2+}$ (see below).

**Properties of MGA synthase and DGA synthase.** Both MGA synthase and DGA synthase were stimulated by the addition of K$^+$ ions, displaying maximum activities at a concentration of 0.5 M (Fig. 5). The optimal pH for both reactions was 8.0 (Fig. 6). The addition of arachidonylceramid was essential for the activities of both MGA and DGA synthase (Table 2).

The two activities were different in terms of the effect of Mg$^{2+}$. DGA synthase required 10 mM Mg$^{2+}$ for maximum activity (Fig. 7A), while MGA synthase did not require the addition of Mg$^{2+}$ ions. Figure 7B and C showed the effect of Mg$^{2+}$ concentration on the proportion of the products (MGA and DGA) of the MGA synthase reaction using archaeol as a substrate. When no Mg$^{2+}$ was added to the reaction mixture containing either fraction AS or fraction BM as an enzyme source, almost all products were MGA, and only a trace amount of DGA was formed. This indicates that the effect of Mg on the MGA synthase activity was not due to the state of the enzymes (whether these were membrane-associated or not). The pattern of total glucose incorporation into lipid varied depending on the enzyme source. In the case of the MGA synthase assay of fraction AS from archaeol as a substrate, less glucose was incorporated into lipid as the Mg$^{2+}$ ion concentration increased (Fig. 7B), while more glucose was incorporated when fraction BM was used as an enzyme source for the same assay (Fig. 7C). This difference is mainly derived

![FIG. 3. Negative ion FAB mass spectra of MGA (A) and DGA (B) enzymatically prepared from archaeol and MGA, respectively.](http://jb.asm.org/)

![FIG. 4. 1H-NMR spectra of MGA (A) and DGA (B) (500 MHz in CDCl$_3$ for MGA and in CDCl$_3$-CD$_3$OD (7:3) for DGA).](http://jb.asm.org/)
from the difference in the proportion of activity of MGA synthase and DGA synthase in the fractions. In fraction AS, DGA synthase has one-third of the activity of MGA synthase (Table 1). On the other hand, DGA synthase has an activity two times higher than that of MGA synthase in fraction BM (Table 1). The difference in the Mg\(^{2+}\) ion requirement for the activities discriminates MGA synthase and DGA synthase. That is, it is concluded that these two activities are effected by two different enzymes. MGA synthase activity in the homogenate containing some DGA synthase can be expected to be assayed without interference with DGA synthase activity in the absence of the Mg\(^{2+}\) ion. In fact, when archaeol and UDP-[U-\(^{14}\)C]glucose were reacted in the absence of Mg\(^{2+}\) with fraction AS, which is the condition of the MGA synthase assay, 94% of the incorporated \(^{14}\)C-glucose appeared in MGA and 6% appeared in DGA. Considering that DGA is synthesized via MGA from archaeol and that DGA has two glucose units, this corresponds to a product proportion of 97 mol% MGA and 3 mol% DGA. At 10 mM Mg\(^{2+}\), the proportion of DGA formed from archaeol increased to approximately 25 mol% of the total products (Fig. 7B).

A fairly small amount of DGA was synthesized without the addition of Mg\(^{2+}\) (Fig. 2A and B). This is determined according to the presence of a small amount of MGA\(^{2+}\) in the cell homogenates. Ca\(^{2+}\) was also effective, but less so than Mg\(^{2+}\), on the activity of DGA synthase. The addition of 5 or 10 mM Ca\(^{2+}\) showed 20% of the Mg\(^{2+}\) effect on DGA synthase activity. Addition of 1 mM EDTA to the assay mixture completely abolished DGA synthesis from MGA.

**Mode of reaction of DGA synthase.** Chloroplasts of plant cells have galactolipid:galactolipid galactosyltransferase (EC 2.4.1.184), which catalyzes an interlipid exchange of galactose between two molecules of monogalactosyldiacylglycerol and leads to the formation of digalactosyldiacylglycerol and diacylglycerol (8). This kind of activity was examined as follows. \(^{14}\)C]MGA enzymatically prepared by the MGA synthase reaction was incubated with cell homogenates in the presence or absence of nonradioactive UDP-glucose. \(^{14}\)C]DGA was synthesized only in the presence of UDP-glucose (Fig. 8). This result indicates that DGA synthase is MGA:UDP-glucose glucosyltransferase and not glucosylarchaeol:glucosylarchaeol glucosyltransferase.

**Substrate specificity of MGA synthase.** Archaeal glycolipids and phospholipids are primarily characterized by the stereoconfiguration of the glycerol backbone on which two hydrocarbon chains are bound at the sn-2 and -3 positions (sn-2,3 stereostructure). Intermediates of the phospholipid biosynthesis pathway in archaea have unsaturated hydrocarbon chains, which are then saturated to the final form in the membrane. The structural requirements for the substrate of the enzymes involved in glycolipid biosynthesis are, therefore, of special interest. We examined the activities of MGA synthase towards a variety of chemically synthesized substrate analogs, including archaeol analogs with sn-1,2 stereostructure or unsaturated hydrocarbons, and tetraether-type lipids (Table 3). Ordinary saturated archaeol was the most active substrate for MGA synthase. The stereostructure of the glycerol backbone was critical to MGA synthase activity; the synthetic enantiomeric saturated archaeol with an sn-1,2 stereostructure showed quite

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**TABLE 1. Enzyme activity of each cell fraction**

<table>
<thead>
<tr>
<th>Fraction</th>
<th>MGA synthase</th>
<th>DGA synthase</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Total activity (nmol/h)</td>
<td>%</td>
</tr>
<tr>
<td>AS</td>
<td>4,057</td>
<td>93</td>
</tr>
<tr>
<td>AW</td>
<td>61</td>
<td>1</td>
</tr>
<tr>
<td>AM</td>
<td>248</td>
<td>6</td>
</tr>
<tr>
<td>BS</td>
<td>3,146</td>
<td>57</td>
</tr>
<tr>
<td>BW</td>
<td>1,015</td>
<td>19</td>
</tr>
<tr>
<td>BM</td>
<td>1,311</td>
<td>24</td>
</tr>
</tbody>
</table>

* Fractions AS (supernatant), AW (membrane washings), and AM (membrane) were prepared in homogenate A at pH 7.5, and fractions BS (supernatant), BW (membrane washings), and BM (membrane) were prepared in homogenate B at pH 6.5.

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**FIG. 5. Effects of K\(^{+}\) concentration on MGA synthase activity and DGA synthase activity.**

**FIG. 6. Effects of pH on MGA synthase activity (A) and DGA synthase activity (B).**
low activity compared with saturated archaeol with an sn-2,3 stereostructure. Unsaturated archaeol with a natural sn-2,3 stereostructure exhibited low activity (29%) compared with saturated natural archaeol. It is concluded that MGA synthase recognizes the stereostructure of the glycerol backbone and saturation of the hydrocarbon chains.

Tetraether lipids (caldarchaetidylinositol and caldarchaeol) exhibited activities similar to that of the saturated natural diether lipid when incubated with fraction AS in the absence of Mg\(^{2+}\) (Table 3). Even when archaeidylinositol was omitted from the reaction mixture, caldarchaetidylinositol exhibited a similar activity as in the presence of archaeidylinositol because caldarchaetidylinositol has the same polar group as archaeidylinositol (phosphoinositol). The chloroform-soluble \(^{14}\)C-labeled products from caldarchaetidylinositol displayed two spots on TLC, with solvent B comigrating with authentic MGCI and DGCI (\(R_f\) values of 0.27 and 0.09, respectively).

Table 4 shows the proportion of the products from caldarcharctidylinositol as a substrate with the soluble or membrane fraction as an enzyme in the presence or absence of archaetidylinositol and Mg\(^{2+}\). The addition of Mg\(^{2+}\) stimulated the synthesis of diglucosyl caldarchaetidylinositol, as seen in the case of DGA synthesis from archaeol (Fig. 7B and C). Since caldarchaeol has two hydroxyl groups per molecule, similar activity was exhibited even if the concentration of the lipid substrate was lowered to half of the standard assay condition (0.1 mM) (Table 3). The chloroform-soluble \(^{14}\)C-labeled products from caldarchaeol displayed four spots on TLC with solvent A. The two main spots comigrated with chemically synthesized monoglucosyl caldarchaeol (\(R_f = 0.87\)) and 1,1’-diglucosyl caldarchaeol (caldarchaeol with a glucose unit on each hydroxyl group) (\(R_f = 0.68\)). The other two products may be triglucosylcaldarchaeol with two glucose moieties on one side and one glucose moiety on the other side and tetraglucosylcaldarchaeol with two glucose moieties on both sides, although these were not experimentally verified. The ratio of monoglucosyl caldarchaeol, 1,1’-diglucosyl caldarchaeol, and the other products was 55:39:6.

**DISCUSSION**

The activities of MGA synthase and DGA synthase were found in *M. thermoautotrophicus* homogenates. The two enzymes catalyzed transfer of a glucose unit from UDP-glucose to archaeol and MGA to form MGA and DGA, respectively. This is the first report on the in vitro biosynthesis of glycolipids in archaea. It is likely that the two activities are carried out by two separate enzymes based on the following observations. (i) Although the two enzymes were loosely associated with the membranes, they are easily released from the membranes during cell disruption in buffer solutions at different rates. DGA synthase was less easily released from the membrane than MGA synthase. (ii) DGA synthase required Mg\(^{2+}\) for the activity but MGA synthase did not. In *Bacillus subtilis*, one enzyme catalyzes these two steps of glycosylation in contrast to the archaeal enzymes (11). We used fraction AS as a source of MGA synthase, which contained 93% of the total activity of MGA synthase with the highest specific activity among the homogenate fractions. For DGA synthase we used fraction

![FIG. 7. Effects of Mg\(^{2+}\) concentration on DGA synthase activity (A) and (B and C) MGA synthase assay by use of the supernatant fraction AS (B) and the membrane fraction BM (C) of *M. thermoautotrophicus* homogenate as an enzyme source. Glc, glucose.](http://jb.asm.org/)

**Table 2. Effects of archaetidylinositol on MGA synthase activity and DGA synthase activity**

<table>
<thead>
<tr>
<th>Assay and lipid substrate</th>
<th>AI(^a)</th>
<th>Relative activity (mean ± SE)(^b)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MGA synthase activity</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Archaeol</td>
<td>+</td>
<td>100</td>
</tr>
<tr>
<td>None</td>
<td>-</td>
<td>4.8 ± 0.6</td>
</tr>
<tr>
<td>DGA synthase activity</td>
<td></td>
<td></td>
</tr>
<tr>
<td>MGA</td>
<td>+</td>
<td>100</td>
</tr>
<tr>
<td>None</td>
<td>-</td>
<td>4.3 ± 0.7</td>
</tr>
</tbody>
</table>

\(^a\) AI, archaetidylinositol. +, present; -, absent.

\(^b\) Data are from duplicate assays.
BM, which contained 52% of the total activity of DGA synthase with the highest specific activity among the homogenate fractions. Although these fractions represent each of the particular enzyme activities, each fraction still contained the other enzyme. However, the activities of MGA synthase and DGA synthase can almost be determined separately with or without the addition of Mg\(^{2+}\) ions, even though the enzymes were not completely separated from each other.

A high concentration (0.5 M) of K\(^+\) ions was also necessary for both enzyme activities. This corresponds with the intracellular concentration of the K\(^+\) ion (0.62 to 0.78 M) in *M. thermotaurothrophicus* (35). The K\(^+\) ion dependence of the polar lipid biosynthetic enzyme in this methanocarchaeon has been also observed in CDP-archaeol synthase (25). Although the intracellular pH (6.7) (10) is not optimal for activity, both enzymes are still active at pH 6.7. An anionic phospholipid (archaeetidylinositol) was essential for both enzyme activities. In the case of bacterial glycolipid synthases, the monoglucosyl diacylglycerol (MGDG) synthase of *Acholeplasma laidlawii* is absolutely dependent upon the presence of anionic phospholipids such as phosphatidylglycerol (5, 13). This seems to constitute a rate-keeping connection between the archaeetidylinositol and glucolipid pathways in vivo, which yields the constant surface charge density of the lipid bilayer membrane as discussed by Christiansson et al. (4, 37). Although the outline of the mechanism of archaenal glycolipid synthesis is the same as the bacterial one, there are some differences in enzyme properties among species. Both enzymes of *Acholeplasma* require detergent and Mg\(^{2+}\) ions, while both enzymes of *Methanothermobacter* did not require any detergent and the first enzyme (MGA synthase) did not require Mg\(^{2+}\). MGA synthase was inhibited by 0.2 or 1% Triton X-100. Because, according to the data of Sprott and Jarrell (35), the intracellular Mg\(^{2+}\) concentration in *M. thermotaurothrophicus* increases from 1.4 mM (mid-log phase) to 12 mM (stationary phase) as the cultures aged, it is presumed that DGA synthase activity may vary several times during its growth. In fact, gentiobiose as a polar head group of polar lipid of this methanoarchaeon increased from 43% to 50% between the log and stationary phases of growth, while other polar groups of its phospholipids decreased during the same period (24).

2,3-Di-\(O\)-geranyleraneryl-sn-glycerol-1-phosphate (unsaturated archaeetic acid, DGGGP) is an important intermediate in archaenal phospholipid biosynthesis (25). Although it is not yet known how saturated archaeol, which is the most active substrate of MGA synthase, is formed in vivo, it is possibly made via this unsaturated intermediate (DGGGP). In order to determine the real substrate for glycolipid biosynthesis, we investigated the substrate specificity. MGA synthase preferred fully saturated archaeol (2,3-di-\(O\)-phenyl-archaeol) to fully unsaturated archaeol (2,3-di-\(O\)-geranyleraneryl-archaeol). On the other hand, archaeetidylserine synthase of *M. thermotaurothrophicus* exhibited similar activities when CDP-unsaturated archaeol and CDP-saturated archaeol were used as substrates (23). Nishimura and Eguchi have reported that 2,3-di-\(O\)-geranyleraneryl-sn-glycerophospholipid reductase is detected in cell extracts of *Thermoplasma acidophilum* (30). Similar geranyleraneryl reductase was also reported in *Archaeoglobus fulgidus* by Murakami et al. (26).

![FIG. 8. Requirement of UDP-glucose for DGA synthase activity. DGA synthase reactions were carried out with \(^{14}C\)MGA in the presence (left lane) or absence (right lane) of nonradioactive UDP-glucose. The products were analyzed by one-dimensional TLC. An autoradiogram of the TLC was shown.](image)

**TABLE 3. Substrate specificity of MGA synthase**

<table>
<thead>
<tr>
<th>Compound</th>
<th>Lipid substrate</th>
<th>Hydrocarbon</th>
<th>Stereostereosstructure</th>
<th>Concn (mM)</th>
<th>Archaeetidylinositol concn (mM)</th>
<th>% Relative activity (mean ± SE)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saturated archaeol</td>
<td>Phytanyl</td>
<td>sn-2,3</td>
<td>0.2</td>
<td>0.2</td>
<td>100</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Phytanyl</td>
<td>sn-1,2</td>
<td>0.2</td>
<td>0.2</td>
<td>8.6 ± 0.1</td>
<td></td>
</tr>
<tr>
<td>Unsaturated archaeol</td>
<td>Geranylgeranyl</td>
<td>sn-2,3</td>
<td>0.2</td>
<td>0.2</td>
<td>29 ± 1</td>
<td></td>
</tr>
<tr>
<td>Caldaetidylinositol</td>
<td>Biphytanyl</td>
<td>sn-2,3</td>
<td>0.2</td>
<td>0.2</td>
<td>84 ± 2</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Biphytanyl</td>
<td>sn-2,3</td>
<td>0.2</td>
<td>0.2</td>
<td>92 ± 5</td>
<td></td>
</tr>
<tr>
<td>Caldaetanol</td>
<td>Biphytanyl</td>
<td>sn-2,3</td>
<td>0.2</td>
<td>0.2</td>
<td>100 ± 7</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Biphytanyl</td>
<td>sn-2,3</td>
<td>0.1</td>
<td>0.2</td>
<td>94 ± 13</td>
<td></td>
</tr>
</tbody>
</table>

\(a\) The conditions of the experiments were the same as described in the legend to Fig. 2 for 1 h of incubation using various substrate compounds.

\(b\) Data are from duplicate assays.
These enzymes exhibit broad substrate specificity; hydrogenation of unsaturated phospholipids can occur before or after the attachment of polar head groups. In the psychrotrophic archaeon *Halobacterium halochloris*, unsaturated lipids were detected in trace amounts in cells grown at 20°C but at 12°C accounted for 70% of the phospholipids and 10% of the glycolipid (9), possibly as a result of incomplete saturation of unsaturated precursors. These in vivo results, in combination with our in vitro results, suggest that while phospholipids are hydrogenated after attachment of polar head groups, the reduction step (hydrogenation) of the unsaturated intermediate may occur before the attachment of the glucose moiety in glycolipid (MGA) biosynthesis. It is worth noting that the hydrogenation step in glycolipid synthesis is different from that in phospholipid synthesis. The real substrate of MGA synthase in this methanoarchaeon is probably saturated archaeol. The tetraether-type phosphoglycolipid was synthesized as shown in Fig. 9. This finding is consistent with the conclusion from in vivo experiments in *Thermoplasma acidophilum* using terbinafine, an inhibitor of tetraether lipid biosynthesis (27). It is suggested that sugar residues are attached to the tetraether lipid core after the condensation of two molecules of diether-type phospholipid and removal of one phosphate-containing polar head group. The fact that diether-type phospholipid but not diether-type glycolipid accumulated during terbinafine inhibition (27) can be explained by the difference in the synthetic mechanisms of tetraether-type phospholipids and glycolipids.

The mode of glucose transfer reaction of MGA synthase is similar to that of 1,2-diacylglycerol 3-glucosyltransferase (MGDG synthase) from *A. laidlawii*. That is, glucose is transferred from UDP-glucose to diradylglycerol core lipid. The requirement of anionic phospholipid is also similar. A different enzyme catalyzes transfer of the second glucose moiety. 

![Possible pathways of DGCI synthesis from caldarchaetidylinositol](http://jb.asm.org/)

**FIG. 9.** Possible pathways of DGCI synthesis from caldarchaetidylinositol. G, glucose.

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**TABLE 4.** Product composition for reactions of caldarchaetidylinositol and UDP-glucose with fraction AS or BM under conditions similar to those of MGA synthase reaction with or without Mg2+

<table>
<thead>
<tr>
<th>Archaeodlysinositol</th>
<th>Mg2+ concn (mM)</th>
<th>Enzyme fraction</th>
<th>Product (mol%)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>−</em></td>
<td>0</td>
<td>AS</td>
<td>91</td>
</tr>
<tr>
<td><em>−</em></td>
<td>5</td>
<td>AS</td>
<td>84</td>
</tr>
<tr>
<td>+</td>
<td>0</td>
<td>AS</td>
<td>87</td>
</tr>
<tr>
<td>+</td>
<td>5</td>
<td>AS</td>
<td>56</td>
</tr>
<tr>
<td>+</td>
<td>0</td>
<td>BM</td>
<td>100</td>
</tr>
<tr>
<td>+</td>
<td>10</td>
<td>BM</td>
<td>96</td>
</tr>
</tbody>
</table>

*See Table 1.

Archaeodlysinositol was omitted in an assay mixture, because caldarchaetidylinositol has the same polar group as archaeodlysinositol.

1 mM EDTA was added to chelate divalent cations.
**ACKNOWLEDGMENTS**

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**REFERENCES**


