GLYCERYL ETHER PHOSPHOLIPIDS IN SELENOMONAS RUMINANTIUM

YOSHIYUKI KAMIO, KYO CHANG KIM, AND HAJIME TAKAHASHI

Department of Agricultural Chemistry, Faculty of Agriculture, Tohoku University, Sendai 980

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Glyceryl ether containing phospholipid was found to exist in a strictly anaerobic bacterium, Selenomonas ruminantium. Glyceryl ether fraction was obtained from total phospholipid by LiAlH₄ and methanol-HCl treatments followed by column chromatography with silicic acid. Glyceryl ether was identified by thin-layer chromatography and infrared absorption spectrum. The molar percentage composition of glyceryl ether in total phospholipids was approximately 3. When labeled valerate or caproate was supplemented to a glucose medium, the radioactivity was incorporated into fatty alcohols of glyceryl ether side chains. The major radioactive components of glyceryl ether from ¹⁴C-valerate grown cells were C₁₁:₁, C₁₂:₀, and C₁₃ fatty alcohols. When cells were grown with ¹⁴C-caproate, the major radioactive glyceryl ether contained C₁₂:₀, C₁₂:₁, and C₁₃ fatty alcohols.

It has been reported from this laboratory (1) that Selenomonas ruminantium var. lactilytica, a strictly anaerobic bacterium isolated from sheep rumen contents, contains plasmalogens as components of phospholipid. It is also noted (2) that S. ruminantium requires a normal saturated volatile fatty acid for its normal growth in a glucose medium. No such obligate requirement is observed when the organism is cultured in a lactate medium. Further studies (1, 3) have shown that ¹⁴C-labeled fatty acid is incorporated into fatty acid and aldehyde moieties of phospholipid fraction.

Glyceryl ether phospholipid has been known to occur in animal sources including bovine (4), porcine (5), and rabbit bone marrows (6), bovine erythrocyte (7), and two species of terrestrial slug (8). These tissues and organisms also contain appreciable amounts of plasmalogens (4–8). These results led us to examine the presence of glyceryl ether phospholipid in S. ruminantium. The present paper demonstrates the occurrence of glyceryl ether phospholipids in S. ruminantium. Chemical nature of the glyceryl ether side chains is also described with reference to growth conditions.

1 Postgraduate student of the Tohoku University on leave from Chung Buk College of Korea.
MATERIALS AND METHODS

Organisms. *Selenomonas ruminantium* var. *lactilytica*, described in an earlier paper (2), was also used in the present study.

Media and cultural conditions. The organism was grown in the yeast extract-glucose medium supplemented with either valerate (C₅) or caproate (C₆) at the final concentration of 0.0019%. When lactate was used as a carbon source, the yeast extract-biotin medium was employed. The detail of the compositions of these medium was described previously (2).

The organism was grown in the glucose medium for 8 hr or in the lactate medium for 18 hr at 37° under anaerobic conditions. Cells were harvested by centrifugation, washed twice with water, and stored overnight in a deep freezer prior to the extraction of lipids.

Extraction of phospholipids. Packed cells from a 2-liter or a 40-liter culture were extracted three times with ethanol-ether (3:1) and then three times with chloroform-methanol (1:3). Extracts were combined, evaporated to dryness under reduced pressure, and re-extracted with ether. Each lipid sample was applied to a silicic acid column (1.5 x 10 cm for 2-liter culture or 3.2 x 35 cm for 40-liter culture). After neutral lipids were eluted from the column with chloroform, the phospholipid fraction was eluted with chloroform methanol (1:1). This fraction was concentrated in vacuo and subjected to further analysis.

Detection and determination of glyceryl ether phospholipids. Quantitative test for glyceryl ether phospholipids was carried out by the method of THOMPSON and LEE (9) as follows. LiAlH₄ (300 mg) was suspended in 15 ml of water-free ether and the suspension was placed in a 250-ml Erlenmeyer flask. The flask was immersed in a cold bath of Dry Ice and acetone. Approximately 50 mg of phospholipid in 10 ml of dehydrated ether was added dropwise with mechanical stirring. After the addition of lipid, the flask was brought slowly to room temperature and then refluxed for 30 min. Finally, excess LiAlH₄ was decomposed with water at Dry Ice-acetone temperature. This treatment cleaves all the ester linkages of phospholipid. Thus, fatty acid moieties are released and reduced to fatty alcohols, and the phosphate group is also released from glycerol skeleton. Plasmalogens are converted into cyclic acetals but the glyceryl ether linkages are not affected.

The ether-soluble reaction products which contained glyceryl ether, cyclic acetal, and fatty alcohol were chromatographed over a silicic acid column (1.5 x 10 cm) by the method of HANAHAN and WATTS (7). The fatty alcohols were eluted first with hexane-ether (3:1) and then cyclic acetals and glyceryl ethers were eluted with hexane-ether (1:3). Analysis of lipid material in the latter fraction was carried out by thin-layer chromatography with silica gel H (E. Merk, Darmstadt, Germany) as a layer of approximately 250 μ thickness. Solvent system was composed of light petroleum (bp 30–60°), ether, and acetic acid (30:70:1). The spots were detected by spraying 50% H₂SO₄...
and heating at 180° for 10 min. As a standard, chimyl alcohol from dogfish liver (Sigma Chemical Co., U.S.A.) was used.

Quantitative determination of glyceryl ether was carried out by the following method. The treatment of phospholipid with LiAlH₄ was carried out as above. The ether-soluble reaction products containing glyceryl ether, cyclic acetal, and fatty alcohol were evaporated to dryness under reduced pressure. In order to cleave the cyclic acetal linkage, 2 ml of methanol containing 2 N HCl was added and kept for 10 min at room temperature. The acid hydrolysis also hydrolyzes vinyl ether linkage of deacylated plasmalogens, which might be contaminated in the above fraction. After addition of 5 ml of water, ether-soluble products were extracted three times with ether and washed twice with water. Ether was evaporated, the residue was dissolved in hexane-ether (3:1), and applied to a silicic acid column (1.5x10 cm). After fatty alcohols were eluted with hexane-ether (3:1), glyceryl ether fraction was eluted with hexane-ether (1:3). Glycerol in the latter fraction was estimated by the method of HANAHAN and OLLEY (10) as follows. To the sample in 2 ml of water were added 0.1 ml of 10 N H₂SO₄ and 0.5 ml of 1 M NaIO₄. The mixture was kept at room temperature for 5 min and then 0.5 ml of 10% NaHSO₃ was added. To the solution (1 ml) was added 5 ml of 0.18% chromotropic acid in 19.6 N H₂SO₄ and heated for 30 min in a boiling water bath. When cooled, 0.5 ml of half saturated thiourea solution was added. Absorbancy was measured by a Hitachi FPW-4 colorimeter equipped with a 55 filter (550 mµ). The amount of glyceryl ether was expressed as chimyl alcohol equivalent.

**Analysis of glyceryl ether side chains as 2,3-O-isopropylidene derivatives.** Glyceryl ethers obtained as above were converted to isopropylidene derivatives as described by HANAHAN et al. (11). Samples (25 mg) were suspended in 5 ml of acetone and 0.025 ml of 12 N HClO₄ was added. After 20 min, water was added until the solution became turbid. The mixture was extracted with ether. The ether fraction was washed three times with water. After the solvent was evaporated in vacuo, the residue was dissolved in hexane. This solution was placed on a silicic acid column (1.5x20 cm). Isopropylidene derivatives were eluted from the column with 250 ml of hexane-ether (9:1). After the solvent was evaporated, the derivatives were analyzed by gas-liquid chromatography as will be described later.

**Incorporation of labeled precursors in glyceryl ethers.** Sodium valerate [1-¹⁴C] was purchased from the Daiichi Kagaku Yakuhin Co., Tokyo, and sodium caproate [1-¹⁴C] was obtained from the Calbiochem, U.S.A. ¹⁴C-Valerate (20 µCi and final concentration of 0.0019%) or ¹⁴C-caproate (20 µCi and final concentration of 0.0019%) was added to 500 ml of the glucose-yeast extract medium. After grown in these media for 9 hr, the cells were harvested and phospholipid fractions were prepared. Glyceryl ether fractions, obtained after the LiAlH₄ treatment and silicic acid chromatography as described above, was chromatographed on thin-layer plates. The radioactive spot, correspond-
ing to glyceryl ethers, was eluted with hexane-ether (1:3). A portion of this sample was counted with a liquid scintillation spectrometer (GSL-112 B, Kobe Kogyo Co., Kobe). The remaining sample was converted to isopropylidene derivatives and analyzed by a radio-gas chromatograph equipped with a thermal conductivity detector for chemical analysis and a gas-flow proportional rate meter for radioactivity measurement (Model RD-1, Yanagimoto Seisakusho, Kyoto). A column of Diasolid L (60–80 mesh, Nippon Kuromoto Kogyo, Tokyo) containing 10% poly(diethylene glycol) succinate (Nippon Kuromato Kogyo, Tokyo) was employed with He as a carrier gas. The operating temperature of the columns (3 mm x 3 m) was 190°. Each peak in the effluent was identified by the relative retention time which was obtained with authentic isopropylidene derivatives of chimyl alcohol and batyl alcohol (Sigma Chemical Co., U.S.A.). The chemical and radioactive percentages of each component were calculated from the ratio of the areas of its peak to the total areas of all the peaks.

Infrared spectrophotometric analysis of glyceryl ether. The glyceryl ether fraction, prepared by LiA1H₄ treatment, HCl hydrolysis, followed by chromatography, was converted to isopropylidene derivatives. The derivatives were spread on a NaCl cell, and the absorption spectrum was measured with an infrared spectrophotometer (Model IR-E, Nippon Bunko Kogyo, Tokyo).

Other analytical methods. The quantitative determination of phosphate in phospholipid was carried out by the KING method (12) after HClO₄ digestion. The amount of phosphate was expressed as P.

RESULT

Occurrence of glyceryl ether phospholipid in S. ruminantium cells

Table 1 summarizes the amounts of glyceryl ether and total phosphorus in the phospholipid fraction of the cells grown in lactate or glucose medium. The amount of glyceryl ether phospholipids was relatively small, being approximately 3% of total phospholipids.

Thin-layer chromatography and infrared absorption spectrum of the glyceryl ether fraction

Cells were grown in the lactate medium supplemented with biotin.

Table 1. Amounts of glyceryl ether and phosphorus in phospholipid from S. ruminantium.

<table>
<thead>
<tr>
<th>Energy sources</th>
<th>Glyceryl ether (µmole/mg dry weight of cells)</th>
<th>Phosphorus (µmole/mg dry weight of cells)</th>
<th>Molar ratio GE/P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lactate</td>
<td>0.89</td>
<td>26.1</td>
<td>0.034</td>
</tr>
<tr>
<td>Glucose</td>
<td>0.35</td>
<td>17.0</td>
<td>0.026</td>
</tr>
</tbody>
</table>
Phospholipids were extracted, treated with LiAlH₄, and after chromatography the hexane-ether (1:3) fraction was prepared. Thin-layer chromatograms of this fraction and authentic chimyl alcohol are shown in Fig. 1. As shown, two major spots were detected. Spot A was identified as glyceryl ether by the identity of its Rf value with that of authentic chimyl alcohol. Moreover, the spot A compound was not separated from chimyl alcohol upon cochromatography. The spot B compounds were presumed to be a cyclic acetal derived from plasmalogen.

The most purified glyceryl ether fraction prepared from *S. ruminantium* cells was analyzed by infrared spectrophotometry. The absorption spectra of this sample and that of authentic chimyl alcohol are shown in Fig. 2. The characteristic absorption bands of the primary and secondary ether bonds (-CH₂-O-CH₂- and >C-O-C\), respectively, which appeared between 1060 and 1130 cm⁻¹, were observed in both samples. Two absorption bands of branched
methyl linkage (-CH\textsubscript{3}) were also observed in both samples. Absorption at 960 cm\textsuperscript{-1} of sample b appears to be due to double bonds (-C=\textsuperscript{\textndash}C-) (cf. Table 3). This absorption band was not significant with chimyl alcohol (main fatty alcohol: C\textsubscript{16:0}). These results further supported the presence of glyceryl ether phospholipids in \textit{S. ruminantium} cells.

Incorporation of labeled valerate and caproate into lipid materials

Cells were grown in the glucose medium supplemented either valerate [\textsuperscript{1\textsuperscript{14}C}] or caproate [\textsuperscript{1\textsuperscript{14}C}]. After the cells were harvested and washed, phospholipid fraction was prepared. As shown in Table 2, most of the incorporated radioactivity was present in the phospholipid fraction. The glyceryl ether fraction, isolated as described above, was also radioactive. Approximately 3.6\% of incorporated radioactivity in phospholipid fraction was found in the glyceryl ether fraction, in both cases.

Radiochemical purity of glyceryl ether fraction was assayed by thin-layer chromatography. As shown in Fig. 3, both fractions from valerate and caproate grown cells contained single major radioactive spot corresponding to
glyceryl ether. It can be seen, however, that the glyceryl ether fractions contained small amounts of radioactive impurities as minor components. The effect of the treatment of LiAlH₄ products with methanol-HCl before column chromatographic separation of glyceryl ether is also shown in Fig. 3. Cyclic acetals, derived from plasmalogens and which migrated above glyceryl ethers

<table>
<thead>
<tr>
<th>Cell fraction</th>
<th>Cells grown in ¹⁴C-valerate</th>
<th>Cells grown in ¹⁴C-caproate</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Total radioactivity (cpm)</td>
<td>Distribution (%)</td>
</tr>
<tr>
<td>Whole cells</td>
<td>77,100</td>
<td>100</td>
</tr>
<tr>
<td>Phospholipid fraction</td>
<td>57,000</td>
<td>74  100</td>
</tr>
<tr>
<td>Glyceryl ether fraction</td>
<td>2,100</td>
<td>2.7 3.7</td>
</tr>
</tbody>
</table>

Fig. 3. Radiochemical purity of glyceryl ether fraction.
1: Sample from ¹⁴C-caproate grown cells. Without HCl hydrolysis before the column chromatographic separation.
2: Sample from ¹⁴C-caproate grown cells. With HCl hydrolysis before column chromatographic separation.
3: Sample from ¹⁴C-valerate grown cells. With HCl hydrolysis before column chromatographic separation.
Incorporation of labeled valerate and caproate into glyceryl ether side chains

Glyceryl ether fractions from the cells grown with valerate [1-14C] or caproate [1-14C] were converted to isopropylidene derivatives. Percentage compositions of radioactive glyceryl ethers with respect to fatty alcohol components are shown in Table 3. When the cells were grown with labeled valerate, the major glyceryl ether side chain was C11:1, C9:0 and C13 fatty alcohols. When the cells were grown with labeled caproate, however, major fatty alcohol components in glyceryl ethers were C12:0, C12:1 and C10:0.

DISCUSSION

Only a few reports are available on the occurrence of glyceryl ether-containing lipids in microbial cells. SEHGAL et al. (13) have shown the presence of glyceryl ether diphosphatide in an extreme halophile, *Halobacterium cutirubrum*. The present work clearly established the presence of glyceryl ether monophosphatide in a strictly anaerobic bacterium, *Selenomonas ruminantium*. To our knowledge, this is the first paper which pointed out the presence of glyceryl ether monophosphatide in microbial cells. The occurrence of glyceryl ether phospholipids is not confined to *S. ruminantium*. It has

<table>
<thead>
<tr>
<th>No. of carbon atoms in glyceryl ether side chain</th>
<th>Incorporation of radioactivity (%)</th>
<th>^14C-Valerate grown cells</th>
<th>^14C-Caproate grown cells</th>
</tr>
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<tbody>
<tr>
<td>9 : 0</td>
<td>15.5</td>
<td>19.4</td>
<td></td>
</tr>
<tr>
<td>10 : 0</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>11 : 0</td>
<td>9.3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>11 : 1</td>
<td>21.7</td>
<td></td>
<td></td>
</tr>
<tr>
<td>12 : 0</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>12 : 1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>13 : 0</td>
<td>11.5</td>
<td>20.3</td>
<td></td>
</tr>
<tr>
<td>13 : 1</td>
<td>14.5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>14 : 0</td>
<td></td>
<td>7.5</td>
<td></td>
</tr>
<tr>
<td>14 : 1</td>
<td></td>
<td>5.2</td>
<td></td>
</tr>
<tr>
<td>15 : 1</td>
<td>9.5</td>
<td></td>
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<tr>
<td>16 : 0</td>
<td></td>
<td>3.0</td>
<td></td>
</tr>
<tr>
<td>16 : 1</td>
<td></td>
<td>12.7</td>
<td></td>
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<tr>
<td>17 : 1</td>
<td>17.8</td>
<td></td>
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</table>
been found in this laboratory that a number of strictly anaerobic bacteria also contain glyceryl ether phospholipids. These observations will be reported in a subsequent paper.

In a previous paper (1), we have shown that aldehyde-containing phospholipids, plasmalogens, were present in an appreciable amount in *S. ruminantium*. Therefore, three types of phospholipids, namely, diacyl, plasmalogen, and alkoxy (glyceryl ether) glycerophosphatides, were detected in *S. ruminantium*. When the cells were grown in the glucose medium supplemented with valerate, the percentage compositions of diacyl, plasmalogen, and alkoxy glycerophosphatides were 72.6, 25.0, and 2.4%, respectively. RENKONEN (14) showed earlier that similar types of phospholipids were present in ox brain lecithin. The percentage compositions of diacyl, plasmalogen, and alkoxy glycerophosphorylcholines were 95.2, 1.9, and 2.2%, respectively.

When the cells were grown in the yeast extract-glucose medium supplemented with valerate, the major glyceryl ether side chains were C_{11:0}, C_{13:0} and C_{13} fatty alcohols. In caproate-grown cells, the major fatty alcohol components were C_{12:0}, C_{12:1} and C_{10:0}. We have shown previously (3) that, when the cells were grown in the yeast extract-glucose medium supplemented with valerate, the major fatty acid and aldehyde components in total and plasmalogen phospholipid were C_{16:1} and C_{17:1}, respectively. In caproate-grown cells, however, the major fatty acid components in total phospholipids were C_{14:0} and C_{16:0}, while the aldehyde side chain was mainly composed of C_{16:1}. Therefore, it appears that there is no precursor-product relationship between fatty alcohol side chains of glyceryl ethers and fatty aldehyde side chains of plasmalogens.

It has been recently reported by SNYDER et al. (15) that fatty alcohol first reacts with glyceraldehyde 3-phosphate and then is reduced to glyceryl ether by microsomal enzymes from animal sources. BICKERSTAFFE and MEAD (16) have shown that labeled palmitoaldehyde, which is injected into brains of young rats, is incorporated into plasmalogen phospholipids. The data presented in Table 3 demonstrate that glyceryl ether side chains are synthesized with added volatile fatty acids (C_{5} and C_{6}) as precursors. It has been shown previously (3) that fatty aldehyde and acid side chains of phospholipids in *S. ruminantium* are also synthesized with added fatty acids as precursors. Biosynthesis of glyceryl ether and plasmalogen phospholipids in cell-free systems is in progress.

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