Evidence for Covalent Attachment of Diphytanylglycerol Phosphate to the Cell-surface Glycoprotein of *Halobacterium halobium*

Akihiro Kikuchi, Hiroshi Sagami, and Kyozo Ogura

From the Institute for Chemical Reaction Science, Tohoku University, 2-1-1, Katahira, Aoba-ku, Sendai 980-8577, Japan

To understand the biosynthesis of diphytanylglycerolylated proteins, we tried to label halobacterial cells with several radioactive tracers in addition to mevalonic acid because mevalonic acid was, in fact, effectively incorporated into the cells, but the amount of the resulting labeled proteins was too small to be analyzed further. In the course of these experiments, we became aware of the degradation of isoprenyl-modified proteins during cell lysis by the action of endogenous proteases. We improved the analytical method for protein preparation. In this work, we report another novel isoprenoid modification of proteins occurring in this archaeabacterium. We also describe evidence for covalent attachment of this isoprenoid to the halobacterial cell-surface glycoprotein.

**EXPERIMENTAL PROCEDURES**

*Molecular Materials—* (R)-[5-3H]Mevalonic acid, [1-3H]GOH, [1-3H]HFOH, [1-3H]GGOH, [1-3H]GGP, and [1-3H]GGPP (each 15–20 Ci/mmol) were purchased from American Radiolabeled Chemicals. Dolichol (C55), and wheat germ acid phosphatase were purchased from Sigma. Dolichyl diphosphate was chemically synthesized from dolichol (C55) according to the method of Danilov and Chojnacki (7). Dolichols (C60–65) were purified by silica gel column chromatography of dolichols (C60–65) obtained from Ginkgo biloba seeds, followed by reverse-phase C18 column chromatography. Diphytanylglycerol was prepared from *H. halobium* cells according to the method of Minnikin et al. (8) and confirmed by mass spectrometry (9). Trypsin (code no. 109819) and Pronase (code no. 165921) were purchased from Roche Molecular Biochemicals. All other chemicals were from commercially available sources.

*Culture Conditions—* *H. halobium* (R1M1 strain) was a gift from Dr. N. Tokunaga (Osaka University). Cells were grown at 38 °C in an illuminated gyratory shaker (120 rpm) in a growth medium containing 250 g/liter NaCl, 2 g/liter KCl, 20 g/liter MgSO4·7H2O, 0.2 g/liter CaCl2·2H2O, 3 g/liter trisodium citrate, and 10 g/liter bacteriological peptone (Oxoid). The pH was adjusted to 7.4 before autoclaving.

*Metabolic Labeling—* *H. halobium* cells (20 ml) were labeled in the presence of 5 μCi/ml [3H]mevalonic acid, [3H]GOH, [3H]HFOH, [3H]GGOH, [3H]GGP, or [3H]GGPP for 96 h at 38 °C. The cells were collected by centrifugation at 10,000 × g for 10 min at 4 °C and washed with a basalt salt solution (growth medium without peptone), followed by centrifugation for 10 min. The washed cells were lyzed in 5 ml of 10 mM Tris–HCl buffer (pH 7.5) and treated with DNase I (500 units) at 4 °C for 5 min. The proteins in the solution were precipitated by the addition of cold acetone to a final concentration of 80%. The solution was kept at 0 °C for 15 min and centrifuged at 3000 rpm for 20 min. The precipitates were extracted successively three times with acetone/water (4:1), three times with chloroform/methanol (2:1), four times with chloroform/methanol/water (10:10:3), and once with ethanol/water (4:1). The delipidated proteins were dispersed in ethanol/water (4:1) and stored at −20 °C before use. The protein concentration was estimated by the Bio-Rad protein assay method with bovine plasma γ-globulin as a standard.

*Analysis of Delipidated Proteins—* Delipidated proteins were subjected to 12 or 7.5% SDS-PAGE and visualized by CBB or PAS stain. The PAS staining was performed according to a procedure described in the Methods section.

The abbreviations used are: GOH, geraniol; FOH, farnesol; GGOH, geranylgeraniol; GGP, geranylgeranyl monophosphate; GGPP, geranylgeranyl diphosphate; PAGE, polyacrylamide gel electrophoresis; CBB, Coomassie Brilliant Blue; PAS, periodic acid–Schiff; HPLC, high performance liquid chromatography.
previously (10). Radioactive proteins on the gel were detected by auto-
radiography with Fuji RX x-ray film after fluorometric enhancement by EN3HANCE (NEN Life Science Products).

Analysis of Trypsin-digested Peptides—The delipidated proteins ob-
tained from cells labeled with [3H]GOH or [3H]GPP were dissolved in
a solution containing 10 mM MgCl₂, 1 mM dithiothreitol, and 100 mM
Tris·HCl buffer (pH 8.0) and digested with 0.75 mg/ml trypsin at 37 °C
for 12 h. The trypsin-digested peptides were subjected to 12% SDS-
PAGE, and the gel corresponding to radioactive peptides was cut off and
extracted with water at 37 °C. The recovered peptides were treated with
methyl iodide as described (5). They were also incubated with 0.5 M HCl
for 2 h at 4 °C or 95 °C and extracted with chloroform/methanol (2:1).
The radioactive extracts obtained by strong acid treatment were evaporated
under a stream of N₂ and analyzed by normal-phase TLC (Merck
Kieselgel 60) in a solvent system of 1-propanol/NH₄OH/H₂O (6:3:1). The
radioactivity on the plate was detected with a Fuji BAS1000 bioimaging
analyzer. The silica gel corresponding to radioactive materials was
scraped from the TLC plate and extracted with chloroform/methanol
(2:1). The extracts were evaporated under a stream of N₂. Enzymatic
dephosphorylation of the extracts was performed as described previ-
ously (11). Briefly, the sample was suspended in 20 μl of ethanol, and to
the suspension was added 0.1 mg of wheat germ acid phosphatase
containing 200 μl of 50 mM Tris-maleate (pH 6.2) and 0.5% (w/v) octyl
β-D-glycoside. The mixture was incubated at 37 °C for 6 h, and the
hydrolysates were extracted with pentane. The extracts were evapo-
rated under a stream of N₂ and analyzed by normal-phase TLC (Kies-
selgel 60) or reverse-phase C₁₈ TLC (Whatman LKC 18) in a solvent
system of hexane/acetone (20:1, double development) or acetone/water
(40:1), respectively. The positions of standard compounds were detected
by exposing the plates to iodine vapor or by spraying the plates with
10% (w/v) phosphomolybdate/methanol solution. The plates were cut
into 0.5-cm sections along the developed line, and the radioactivity of
the gels corresponding to each section was measured by liquid scintil-
lating counting.

In addition, delipidated proteins of H. halobium cells were separated
by 12% SDS-PAGE after trypsin digestion, electrotransferred to a poly-
vinyldene difluoride membrane, and PAS-stained. The membrane cor-
responding to the PAS stain-positive band (28 kDa) was excised and
subjected to automated Edman degradation.

Analysis by Mass Spectrometry—A sufficient amount of the trypsin-
digested peptides was subjected to 12% SDS-PAGE, and the gel corre-
sponding to 28-kDa peptide bands was cut off and extracted with water
at 37 °C. The recovered peptides were mixed with radioactive 28-kDa peptides. The radioactive materials released by acid treatment of the
mixed peptides were purified by reverse-phase C₁₈ HPLC (YMC, 250 ×
4.6 mm) according to a modified method as described previously (12).
Briefly, the radioactive materials were eluted from the column main-
tained at 45 °C using a solvent system of methanol/isopropyl alcohol
(the isopropyl alcohol contained 10 mM phosphoric acid) (95:5). A pre-
run was necessary to ensure reproducible results. The radioactive frac-
tions were combined and washed with water to remove phosphoric acid.
The purified materials were treated with acid phosphatase, and the
hydrolysates were further purified by normal-phase HPLC (TOSOH,
250 × 4.6 mm) in a solvent system of hexane/acetonitrile (50:1). Mass
spectra of the purified hydrolysates were recorded on a Jeol JMS-
DX600 mass spectrometer (laboratory of Dr. M. Kira, Tohoku Univer-
sity). The potential of the ionizing beam was 70 eV.

RESULTS
Previous work from this laboratory showed that [3H]meval-
onic acid was incorporated into H. halobium cells and that
several proteins with molecular masses between 35 and 45 kDa
were predominantly radiolabeled (5). It was also shown that
19–23% of the total radioactivity in the delipidated proteins
was released in the sulfinium salt cleavage reaction with
methyl iodide and that a diphytanylglycerol group was associ-
ated with these proteins in thioether linkage (13). In the course
of these experiments, we became aware of the fact that appar-
ent differences in protein bands with molecular masses
<35–45 kDa. Halobacteria are known to produce extracellular
protease (13), and Mescher et al. (14) have already reported
that isolated halobacterial cell-surface glycoprotein prepara-
tions contain a protease activity. Therefore, we tested the
action of endogenous protease during protein preparation be-
cause we have used incubation conditions (37 °C, >10 min)
that reduce the viscosity of the cell lysis solution by DNase I
treatment. As shown in Fig. 1A, the high molecular mass protein
bands were reduced with increasing incubation times. Fig. 1B
shows CBB-stained protein bands of [3H]mevalonic acid-la-
beled delipidated protein fraction after 5 min of incubation at
4 °C (lane a) and after >10 min of incubation at 37 °C (lane b),
described in a previous report (5, 6). The fraction in lane b
contained partly degraded proteins. When the same fractions
as in Fig. 1B (lanes a and b) were treated with methyl iodide,
~23% of the total radioactivity of the latter was, in fact, cleav-
able, but that of the former was only 3% released.

To characterize halobacterial isoprenoid-modified proteins, we
again labeled the cells with several radioactive tracers such as
[1-3H]GOH, [1-3H]FOH, [1-3H]GGOH, [1-3H]GPP, [1-3H]G-
GPP, and (R)-[5-3H]mevalonic acid. After labeling with each
radioactive tracer (0.2 μM) for 96 h, delipidated proteins were
prepared without degradation. Fig. 2 (A and B) shows CBB-
stained protein bands and radioactive bands on SDS-PAGE of
delipidated protein fraction, respectively. Incorporation of the
radioactive isoprenoid tracers into delipidated proteins was
greater than that of [3H]mevalonic acid. The major radioactive
band was detected in the region corresponding to 200 kDa,
except in the case of [3H]mevalonate labeling. Since H. halobium
is known to have a characteristic cell-surface glycoprotein with a
molecular mass of 200 kDa (14, 15), we next tried to perform
7.5% SDS-PAGE of each delipidated protein fraction to see the
relationship between the major radioactive protein and the PAS-
stainable glycoprotein. As shown in Fig. 3A, the PAS-stained
glycoprotein bands and radioactive bands on SDS-PAGE of
delipidated protein fraction, respectively. Incorporation of the
radioactive isoprenoid tracers into delipidated proteins was
greater than that of [3H]mevalonic acid. The major radioactive
band was detected in the region corresponding to 200 kDa, and
the radioactivity yields into an organic solvent were
3%. These results suggest that the diphytanylglycerolylated proteins
reported in previous studies (5, 6) are the minor components
among isoprenoid-modified proteins in this halobacterium and
that the major component is the PAS stain-positive cell-surface
glycoprotein. To examine whether the radioactive lipid in the
200-kDa protein is covalently bound to the known 200-kDa glycoprotein, \(^{[\text{3H}] GOH}\)-labeled delipidated proteins were treated with trypsin and analyzed by 12% SDS-PAGE. As shown in Fig. 4A, no protein bands stained with CBB were detected after trypsin digestion. However, a broad radioactive band consisting of two materials was detected in the radioactive hydrolysates moved with a spot of diphytanylglycerol phosphate (17). When the radioactive materials recovered from the TLC plate were treated with acid phosphatase, radioactivity on the plate was detected with a Fuji BAS1000 bioimaging analyzer (lane B). The arrow indicates the position of dolichyl (C\(_{55}\)) phosphate (Dol-P). Ori., origin; S. F., solvent front.

The released materials remained at the origin on a normal-phase TLC plate when a non-polar solvent of benzene/ethyl acetate (9:1) was used, but showed a mobility similar to that of dolichyl (C\(_{55}\)) phosphate under the chromatographic conditions using a polar solvent (Fig. 5). From the chromatographic behavior and chemical properties of the released materials, we speculated that they were non-allylic phosphate ester compounds, such as dolichyl (C\(_{55}\)) phosphate (16) or diphytanylglycerol phosphate (17). When the radioactive materials recovered from the TLC plate were treated with acid phosphatase, –30–40% of the radioactivity of the polar materials was actually detected in pentane-soluble hydrolysates. As shown in Fig. 6, the radioactive hydrolysates moved with a spot of diphytanylglycerol phosphate-modified proteins.

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FIG. 6. Enzymatic dephosphorylation of the materials released by acid treatment of the radioactive peptides. The radioactive PAS-stainable peptides (28 kDa) described under “Experimental Procedures” were incubated in the presence or absence of acid phosphatase. The hydrolysates were analyzed by normal-phase TLC in a solvent system of hexane/acetone (20:1, double development) (A) and by reverse-phase C$_{18}$ TLC in a solvent system of acetone/H$_2$O (40:1) (B). Arrows a–d indicate the positions of authentic standards (C$_{55}$ dolichol, C$_{60}$ dolichol, C$_{65}$ dolichol, and diphytanylglycerol, respectively). Ori., origin; S. F., solvent front.

Fig. 7. Mass spectrograms. The radioactive materials released by acid treatment of the PAS-stainable peptides (28 kDa) were purified by reverse-phase C$_{18}$ HPLC. The purified materials were dephosphorylated by acid phosphatase treatment, and the hydrolysates were further purified by normal-phase HPLC as described under “Experimental Procedures.” A and B, mass spectrograms of the purified hydrolysate and the diphytanylglycerol standard, respectively.
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FIG. 8. Schematic representation of the cell-surface glycoprotein of H. halobium (A) and comparison of protein sequences from the COOH termini of the cell-surface glycoproteins of H. halobium (1), H. volcanii (2), and H. japonica (3) (B). Numbering indicates amino acid positions. M, membrane-binding domain; T, region of a threonine cluster; □, the repeated saccharide unit; ○, N-linked oligosaccharides; ▪, O-linked disaccharides. I and II indicate partial sequences analyzed by Edman degradation. Three repeats of the amino acid sequence motif (D/E)(T/S)₅ and one repeat of the motif TX貔TFTE in H. volcanii (2) are indicated by dashed and solid underlining, respectively. Six repeats of the amino acid sequence motif P TMPro in H. japonica (3) are indicated by underlining. For further explanations, see “Discussion.”

DISCUSSION

This study has demonstrated that the known cell-surface glycoprotein of H. halobium is modified with diphityanglyceryl phosphate. This protein has already been well characterized as the first prokaryotic glycoprotein consisting of 818 amino acids (18), as shown in Fig. 8A. A single high molecular mass saccharide that is composed of repeats of sulfated pentasaccharides is associated with an asparagine residue (position 2). Ten sulfated (HexUA1–4)₂₋₄Glc oligosaccharides are connected to asparagine residues (positions 305, 364, 404, 479, 609, 693, 717, 753, 777, and 781). About 20 Glu1–2Gal disaccharides are O-glycosidically attached to threonine residues, of which 14 threonine residues form a cluster (positions 755–774) adjacent to a stretch of 21 amino acids (positions 795–815) exclusively composed of hydrophobic residues (membrane-binding domain). The highly N- and O-glycosidically modified protein behaves as if it had an apparent molecular mass of 200 kDa on SDS-PAGE. We showed that a PAS stain-positive 200-kDa protein was radioactive when several tritium-labeled isoprenoid compounds (except for [³H]GGHOH) were used as tracers. We could not readily believe in the covalent isoprenoid modification of the glycoprotein because this glycoprotein has been characterized in detail (described above). However, when the trypsin-digested PAS-stained peptides were found to be radioactive, we believed in a modification of the known cell-surface glycoprotein by isoprenoid-derived materials.

Although it is expected that all the saccharides on the 200-kDa protein can be stained with PAS, the majority of the PAS-stained saccharides were recovered in the two trypsin-digested peptides (28 kDa), which corresponded to a C-terminal fragment containing a threonine cluster. The O-linked saccharides might be predominantly stained because sulfated N-linked saccharides may meet with repulsion by negatively charged periodate ions (19). As shown in Fig. 4, the two 28-kDa peptides were apparently radioactive and PAS stain-positive. The mobility difference (~1 kDa) between the two peptides might be due to the degree of glycosylation (20). Electrophoresis of the peptides to a polyvinylidene difluoride membrane also gave two PAS-stained bands, but the bands overlapped each other. The peptides corresponding to the overlapped band were analyzed by Edman degradation and determined to be a mixture of two components. The sequence of the minor component was exactly the same as the N-terminal partial sequence of a fragment of a cell-surface glycoprotein, whereas that of the major one lacked the N-terminal glutamine residue. Since trypsin cleaves peptide bonds involving the carboxyl groups of arginine and lysine, the major peptide fragment lacking glutamine implies the occurrence of an isomeric form that contains either an RNVEIVE or RNVEIVE sequence different from the RNVEIVE sequence found in the known cell-surface glycoprotein.

The linkage between unknown lipids and peptides of the 28-kDa trypsin-digested fragments was resistant to the sulfonium salt cleavage reaction, but was cleavable by acid at 95 °C, yielding non-allylic phosphate ester lipids. Neither phosphate
ester nor pyrophosphate ester lipids were released under the mild acidic conditions, suggesting the presence of a phosphodiester linkage (21). This result led us to speculate that the lipid might be dolichyl (C\(_{55}\)) phosphate because dolichyl phosphate modification of proteins has been already reported in mammalian liver (22), and the carbon chain length of dolichyl phosphate occurring in H. halobium is C\(_{55}\) (16). However, the radioactivity of the hydrolysates released by acid phosphatase treatment co-migrated with diphytanylglycerol on thin-layer chromatography, and the HPLC-purified hydrolysate was identified to be diphytanylglycerol by mass spectrometry. Although the exact type of linkage of diphytanylglycerol to the proteins remains to be established, at present, we conclude that the non-allylic lipid is diphytanylglycerol phosphate from its chromatographic and chemical properties. Coupled with previous findings (5, 6), these results indicate that H. halobium contains two new types of isoprenoid-modified proteins, namely diphytanylglycerol-modified proteins with thioether linkage and diphytanylglycerol phosphate-modified proteins. Two similar types of isoprenoid modification, dolichyl proteins with thioether linkage and dolichyl phosphate-modified proteins, have also been reported in mammalian tissues (22, 23).

The cell-surface glycoproteins of H. halobium, Haloferax volcanii, and Haloarcula japonica have been reported to show a considerable homology in amino acid sequences (18, 24, 25). Fig. 8D shows the protein sequences from the COOH termini of the three cell-surface glycoproteins. The C-terminal membrane-binding domain is conserved in these glycoproteins. However, the cluster of O-glycosylated threonine residues found in H. halobium is not necessarily conserved. In H. volcanii, the threonine cluster is much longer and contains three repeats of the amino acid sequence motif (D/E)(T/S), and a repeat of the motif TTXX(T/S). Six repeats of the amino acid sequence motif PXXTXXE are present in H. japonica instead of the threonine-rich cluster. Kessel et al. (26) have speculated that the unusual structural threonine-rich cluster element serves as a spacer between the membrane-binding domain and the extracellular domain of the cell-surface glycoprotein, thus creating an interspace that may be regarded as analogous to the periplasmic space of gram-negative eubacteria. Therefore, if the modification of the C-terminal domain by diphytanylglycerol phosphate is common to these archaeobacterial glycoproteins and if the hydrophobic diphytanylglycerol group also serves as another membrane anchor, the modification site might be in a limited region adjacent to hydrophobic residues corresponding to the membrane-binding domain. We speculate that the hydroxyl group of the side chain of serine or threonine next to each membrane-binding domain might be a possible modification site. However, if the modification by diphytanylglycerol phosphate is characteristic of H. halobium, the lipid would be associated with a hydroxyl group of either saccharides or the other peptide residues.

Kobayashi et al. (27) have recently reported the presence of glycosylphosphatidylinositol-anchored proteins even in an archaebacterium, Sulfolobus acidocaldarius. Since inositol has not been found in halobacteria (28), the possibility that glycosylphosphatidylinositol-anchored proteins occur in this archaebacterium would be ruled out. In a preliminary experiment, no radioactivity was, in fact, detected in delipidated protein fractions obtained by metabolic labeling with \(^{14}C\)-labeled ethanolamine or inositol (data not shown). The C-terminal hydrophobic peptide chain of the cell-surface glycoprotein is followed by three basic amino acids, as shown in Fig. 8B, indicating that the hydrophobic peptide is not removed (29). Therefore, it is thought that the surface glycoprotein of H. halobium is a novel protein that has not only one single C-terminal hydrophobic peptide chain, but also another hydrophobic lipid chain, unlike mature glycosylphosphatidylinositol-anchored proteins free from the C-terminal hydrophobic peptide of their precursor proteins.

Halophilic archaea lack the peptidoglycan layer, and the cell-surface glycoprotein is the only cell wall component (30). The glycoproteins of H. halobium, H. volcanii, and H. japonica are essential for maintaining the rod shape, the flat disc shape, and the triangular disc shape, respectively (31, 32). Each mature polypeptide contains 818, 794, and 828 amino acids with molecular masses of 86,538, 81,732, and 87,166 Da. These values are much lower than those estimated from SDS-PAGE (200, 170, and 170 kDa, respectively). The same abnormal electrophoretic behavior was found for the deglycosylated surface-layer polypeptide of H. halobium (18). Therefore, these polypeptides may have reduced capacity for SDS binding due to their unusual content of acidic residues such as aspartic acid and glutamic acid (18, 24). In this study, we presented a novel diphytanylglycerol phosphate modification of the halobacterial cell-surface glycoprotein. This modification seems to be common to the glycoproteins of halophilic archaea bacteria and might partly cause the reduced electrophoretic mobility of the glycoproteins on SDS-PAGE. Further experiments are in progress to identify the isoprenoid modification site of the cell-surface glycoprotein of H. halobium.

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