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Identification and Characterization of Geranylgeraniol Kinase and Geranylgeranyl Phosphate Kinase from the Archaebacterium *Sulfolobus acidocaldarius*[1]

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Geranylgeranyl diphosphate is an important precursor of archaebacterial ether-linked lipids, and it has been thought that all of this compound is "*de novo*" synthesized by geranylgeranyl diphosphate synthase. We studied the phosphorylation of geranylgeraniol, which seems to be related to the salvage pathway of biosynthesis of archaebacterial ether-linked lipids, in the Archaebacterium *Sulfolobus acidocaldarius*. Activities of geranylgeraniol kinase and geranylgeranyl phosphate kinase were detected in a cell lysate of *S. acidocaldarius*. The two enzymes were easily separated by ultracentrifugation. The membrane fraction and the cytosolic fraction contained geranylgeraniol kinase activity and geranylgeranyl phosphate kinase activity, respectively. Geranylgeraniol kinase, which requires divalent cation such as Mg^{2+}, Co^{2+}, and Mn^{2+} and NTP (ATP, GTP, CTP, UTP), catalyzes monophosphorylation of (all-*E*)-geranylgeraniol to produce geranylgeranyl phosphate. (all-*E*)-Farnesol, (all-*E*)-hexaprenol, and (all-*E*)-octaprenol were also active as substrates, though they were less effective than (all-*E*)-geranylgeraniol. However, neither geraniol nor (2*E*,6*E*,10*Z*,14*Z*,18*Z*,22*Z*,26*Z*,30*Z*,34*Z*,38*Z*)-undecaprenol was active. This enzyme is extremely thermostable and its pH optimal is between 6.5 and 8.5. The Michaelis constants for (all-*E*)-geranylgeraniol and ATP are 27 nM and 650 μM, respectively.

Key words: Archaebacterium, geranylgeraniol, isoprenoids, kinase, lipid.

Sulfolobus acidocaldarius is a member of Archaea, a recently recognized third kingdom which diverged from Bacteria and Eucarya at an early stage in evolution (*1*). The microorganisms belonging to the third kingdom have several unique characteristics, such as their growth in extreme environments, possession of eukaryotic-like ribosomes, and the structures of modified bases in tRNAs. However, a most interesting feature common to all archaebacteria is the molecular architecture of the lipids in their cellular membrane (*2, 3*).

Archaebacterial membrane lipids are mainly ether-linked lipids in place of the usual ester-linked phospholipids of eubacteria and eukaryotes, which appears to be one of the features enabling the organisms to inhabit extreme environments. These unusual lipids consist of a glycerol unit (or more complex polyols, *i.e.*, nonitocaldarchaeol) and phytane or biphytane units, which are derived from GGPP. GGPP is "*de novo*" synthesized by way of a mevalonate pathway. Some of the enzymes related to this pathway have already been characterized (*4*). Recently we cloned the gene of GGPP synthase from *S. acidocaldarius* and characterized it (*5*). However, the fate of the ether-linked lipids and regulation of the lipid biosynthesis are unclear.

It is well known that, in eubacteria and eukaryotes, free fatty acids are incorporated into cells and converted to acyl-CoA. The CoA derivatives, which are activated forms of fatty acids, are incorporated into membrane lipids and this salvage pathway plays an important role. Poulter *et al.* reported that, in a feeding experiment with the Archaebacterium *Methanospirillum fungatei*, geranylgeraniol was incorporated into 2,3-di-*O*-phytanyl-*sn*-glycerol and 2,3-di-*O*-biphytanyl-*sn*-diglycerol, and they proposed the existence of geranylgeraniol kinase because GGPP was a direct and activated precursor of archaebacterial ether-linked lipids (*6*).

In eubacteria and eukaryotes, three isoprenoid kinases have been identified. Undecaprenyl phosphate and dolichyl phosphate, which are formed by specific kinases, work as sugar-carrier lipids to produce peptideglycan and *N*-glycoprotein, respectively. The kinases play an important role in regulation of the biosynthesis of the above molecules. Recently, farnesol kinase was found from the colonial microalga *Botryococcus braunii* (*7*). However, the isoprenol kinase acting on geranylgeraniol has not been identified.

In order to facilitate research on archaebacterial membrane lipids and to isolate the new enzyme, we have studied phosphorylation of geranylgeraniol by using a cell-free lysate from the Archaebacterium *S. acidocaldarius*.

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Abbreviations: DMAPP, dimethylallyl diphosphate; FPP, farnesyl diphosphate; GGPP, geranylgeranyl diphosphate; GGP, geranylgeranyl phosphate; GGOH, geranylgeraniol; IPP, isopentenyl diphosphate; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate.

EXPERIMENTAL PROCEDURES

Materials—Precoated reversed-phase thin-layer chromatography plates, LKC-18 were purchased from Whatman Chemical Separation. Precoated normal-phase thin-layer chromatography plates, Kieselgel 60 were purchased from E. Merck. ATP was purchased from Oriental Yeast, Tokyo. GTP was purchased from Wako Pure Chemical Industries, CTP and UTP were purchased from Sigma Chemical. (all-*E*)-FPP, (all-*E*)-GGPP, GPP, the strains of *Micrococcus luteus, M. luteus* B-P26, and *Bacillus subtilis* were donated by Dr. Kyozo Ogura, Tohoku University. The strain of *S. acidocaldarius* ATCC 33909 was purchased from the American Type Culture Collection and grown by the method described previously (*5*). [1-^{3}H]Geranyl diphosphate and [1-^{3}H](all-*E*)-FPP were purchased from American Radiolabeled Chemicals. [1-^{3}H]Geraniol and [1-^{3}H](all-*E*)-farnesol were obtained from the diphosphate derivatives by means of alkaline phosphatase hydroxylation. [1-^{14}C]IPP was purchased from Amersham. All other chemicals were of analytical grade.

Synthesis of [1,5,9-^{14}C](all-E)-Hexaprenol—Radiolabeled (all-*E*)-hexaprenol was synthesized by the use of hexaprenyl diphosphate synthase. Hexaprenyl diphosphate synthase was prepared from *M. luteus* B-P26 as described by Fujii *et al.* (*8*). The reaction mixture contained, in a final volume of 100 ml, 20 mM Tris-HCl buffer (pH 7.7), 25 μM geranyl diphosphate, 25 μM [1-^{14}C]IPP (specific activity, 0.342 GBq/mmol), 5 mM $MgCl_2$, 10 mM KF, and the enzyme. After incubation at 37°C for 2 h, the reaction mixture was washed with diethyl ether. Products were extracted with *n*-butanol saturated with H_2O, and the butanol layer was washed with water. The butanol was evaporated by a stream of N_2 gas, and the residues were hydrolyzed according to the method of Fujii *et al.* (*9*). The hydrolysates were extracted with pentane and purified by reversed-phase thin-layer chromatography using LKC-18 developed with acetone/H_2O (19 : 1). The specific activity of [1,5,9-^{14}C](all-*E*)-hexaprenol was 1.03 GBq/mmol.

Synthesis of [1,5,9,13,17-^{14}C](all-E)-Octaprenol—[1,5,9,13,17-^{14}C](all-*E*)-Octaprenol was synthesized by the use of solanesyl diphosphate synthase. Solanesyl diphosphate synthase was prepared from *M. luteus* as described by Ohnuma *et al.* (*10*). The reaction mixture contained, in a final volume of 200 ml, 20 mM Tris-HCl buffer (pH 7.7), 25 μM (all-*E*)-FPP, 25 μM [1-^{14}C]IPP (specific activity, 37.0 MBq/mmol), 5 mM $MgCl_2$, and the enzyme. After incubation at 37°C for 3 h, the reaction products were extracted with 1-butanol saturated with H_2O, and the butanol layer was washed with water. The butanol was evaporated by a stream of N_2 gas, and the residues were hydrolyzed as described above. The hydrolysates, which contained octaprenol and solanesol, were extracted with pentane and purified by reversed-phase thin-layer chromatography as described above. The specific activity of [1,5,9,13,17-^{14}C](all-*E*)-octaprenol was 0.185 GBq/mmol.

Synthesis of [1,5,9,13,17,21,25,29-^{14}C](2Z,6Z,10Z,14Z,18Z,22Z,26Z,30Z,34E,38E)-Undecaprenol—Radiolabeled undecaprenyl diphosphate was synthesized by the use of undecaprenyl diphosphate synthase. Undecaprenyl diphosphate synthase was prepared from *Bacillus stearothermophilus* according to the methods of Koyama *et al.* (T. Koyama *et al.*, unpublished results). The reaction mixture contained, in a final volume of 5 ml, 50 mM Tris-HCl buffer (pH 8.5), 2.5 μM (all-*E*)-FPP, 0.46 μM [1-^{14}C]-IPP (specific activity, 2.07 GBq/mmol), 5 mM $MgCl_2$, 0.25% Triton X-100, 50 mM NH_4Cl, 50 mM 2-mercaptoethanol, and the enzyme. After incubation of the mixture at 55°C for 1 h, the reaction product was extracted, hydrolyzed, and purified as described above. The specific activity of [1,5,9,13,17,21,25,29-^{14}C](2*Z*,6*Z*,10*Z*,14*Z*,18*Z*,22*Z*,26*Z*,30*Z*,34*E*,38*E*)-undecaprenol was 16.6 GBq/mmol.

Synthesis of [1-^{14}C](all-E)-GGOH—Radiolabeled GGPP was synthesized by the use of GGPP synthase. GGPP synthase was prepared from *Escherichia coli* that was transformed with plasmid pGGPS3 as previously described (*5*). The reaction mixture contained, in a final volume of 10 ml, 100 mM succinate-HCl buffer (pH 5.8), 10 μM (all-*E*)-FPP, 9.43 μM [1-^{14}C]IPP (specific activity, 1.92 GBq/mmol), 5 mM $MgCl_2$, and the enzyme. After incubation at 55°C for 2 h, the reaction product was extracted, hydrolyzed, and purified as described above. The specific activity of [1-^{14}C](all-*E*)-GGOH was 1.92 GBq/mmol.

Preparation of Isoprenoid Alcohol Kinase—*S. acidocaldarius* was grown in ATCC medium 1723 at 70°C and harvested according to the method described in the ATCC catalogue. Cells (wet weight, 7.0 g) were suspended in 14 ml of 50 mM Tris-HCl (pH 7.0) containing 10 mM 2-mercaptoethanol and 1 mM EDTA and disrupted by ten sonications, each of 1 min duration, with 5-min intervals in an ice bath, using a Branson Sonifier. The resulting mixture was centrifuged at 6,000×*g* for 20 min at 4°C. The supernatant was further centrifuged at 100,000×*g* for 60 min at 4°C. The supernatant was used as the enzyme fraction (100,000×*g* sup). The precipitate was washed twice with the same buffer. The final precipitate was suspended in the same buffer and used as the enzyme fraction (ppt).

Assay of Isoprenoid Alcohol Kinase—The detergent CHAPS was used to dissolve radiolabeled isoprenols because it showed no effect on either GGOH kinase or GGP kinase activity (data not shown). The reaction mixture contained, in a final volume of 200 μl, 100 mM Tris-HCl buffer (pH 7.0), 10 mM ATP, 0.22 μM [1-^{14}C](all-*E*)-GGOH (specific activity, 1.92 GBq/mmol), 20 mM $MgCl_2$, 17.2 mM CHAPS, and an indicated amount of enzyme. Incubation was carried out for 1 h at 55°C. The reaction mixture was extracted with 1-butanol saturated with water. A part of the butanol extract was directly applied to normal-phase thin-layer chromatography plates, Kieselgel 60. The plates were developed with 2-propanol/NH_4OH/H_2O (6 : 3 : 1), and the radioactivity on the plate was determined with a Bioimaging analyzer BAS2000 (Fuji Film) or a liquid scintillation counter after the gel had been scraped from the plate. Authentic [1-^{14}C]GGP was obtained from [1-^{14}C]GGPP by mild-acid treatment.

RESULTS

Identification of GGOH Kinase and GGP Kinase—A cell lysate of the Archaebacterium *S. acidocaldarius* was prepared by sonication of a cell suspension, followed by centrifugation at 6,000×*g* to remove cellular debris. A portion of the cell lysate was incubated with [1-^{14}C]GGOH, ATP, and Mg^{2+}, and the reaction mixture was extracted

with 1-butanol saturated with water. The extracts were washed with water and analyzed by normal-phase thin-layer chromatography (6 : 3 : 1, 2-propanol/NH_4OH/H_2O).

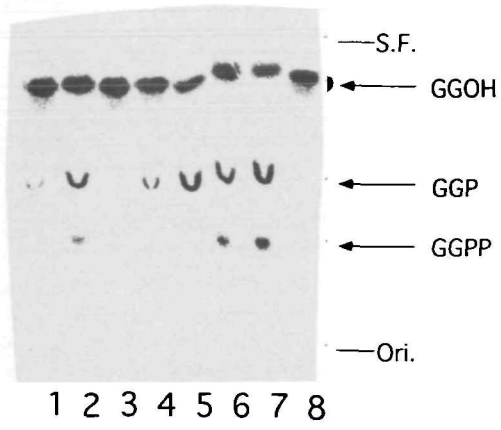

Fig. 1. **Thin-layer chromatograms of the products formed by phosphorylation of GGOH.** Each reaction mixture contained 100 mM Tris-HCl buffer (pH 7.0), 220 nM [1-^{14}C]GGOH (1.92 GBq/mmol), 10 mM ATP, 20 mM $MgCl_2$, 16 mM CHAPS, and the indicated enzyme: lane 1, 6,000×*g* supernatant (100 μg of protein); lane 2, 6,000×*g* supernatant (1 mg of protein); lane 3, 100,000×*g* supernatant (100 μg of protein); lane 4, 100,000×*g* precipitate (100 μg of protein); lane 5, 100,000×*g* precipitate (1 mg of protein); lane 6, 100,000×*g* supernatant (552 μg of protein) and ppt (312 μg of protein); lane 7, 100,000×*g* supernatant (368 μg of protein) and ppt (702 μg of protein); lane 8, none (control). The samples were incubated for 1 h at 55°C and analyzed by thin-layer chromatography as described in "EXPERIMENTAL PROCEDURES."

Enzyme activities of phosphorylation of GGOH to both GGP and GGPP were detected in the cell lysate (Fig. 1, lanes 1 and 2). The cell lysate was subjected to centrifugation at 100,000×*g* for 1 h to obtain a membrane fraction and a cytosolic supernatant. The precipitate fraction was washed with the buffer three times to avoid contamination with soluble enzymes, such as nucleoside diphosphate kinase. Incubation of the 100,000×*g* precipitate with [1-^{14}C]GGOH, ATP, and Mg^{2+} gave only a monophosphorylated product, not a diphosphorylated one (Fig. 1, lanes 4 and 5). No phosphorylated product was obtained from the incubation without either Mg^{2+} or ATP (data not shown). Incubation of the 100,000×*g* supernatant with [1-^{14}C]-GGOH, ATP, and Mg^{2+} gave no product (Fig. 1, lane 3). However, coincubation of the 100,000×*g* supernatant and precipitate yielded both GGP and GGPP (Fig. 1, lanes 6 and 7). These results demonstrate that the formation of GGPP from GGOH is catalyzed by two distinct enzymes, GGOH kinase and GGP kinase, which are obtained in the membrane fraction and cytosol, respectively. GGOH seems to be converted to its monophosphate in the membrane, and this product moves to the cytosol to undergo a second phosphorylation. As shown in Table I, the amount of GGPP formed in experiment 7 (5.96 pmol) was larger than in experiment 6 (3.53 pmol) although the amount of the 100,000×*g* supernatant in experiment 7 (367 μg) was smaller than in experiment 6 (551 μg). These results indicate that the concentration of GGP formed in the reactions is lower than that required to saturate the GGP kinase.

Attempts to solubilize GGOH kinase with a variety of detergents, including Triton X-100, Tween 80, CHAPS,

TABLE I. **Distribution of phosphorylated products formed in the reaction using various subcellular fractions.** Reaction mixtures contained 0.22 μM [1-^{14}C]GGOH (1.92 GBq/mmol), 20 mM $MgCl_2$, 10 mM ATP, 16 mM CHAPS, and indicated subcellular fractions. After a 1-h incubation at 55°C, the amounts of [1-^{14}C]GGP and [1-^{14}C]GGPP were measured as described under "EXPERIMENTAL PROCEDURES."

Experiment No.	Subcellular fraction	Products (pmol)	
		Geranylgeranyl phosphate	Geranylgeranyl diphosphate
1	6,000×*g* sup (100 μg)	1.92	nd[a]
2	6,000×*g* sup (1 mg)	18.6	3.28
3	100,000×*g* sup (100 μg)	nd[a]	nd[a]
4	100,000×*g* ppt (100 μg)	6.86	nd[a]
5	100,000×*g* ppt (1 mg)	31.2	0.54
6	100,000×*g* ppt (312 μg) +100,000×*g* sup (552 μg)	16.4	3.53
7	100,000×*g* ppt (702 μg) +100,000×*g* sup (368 μg)	24.4	5.96

[a]Not detected.

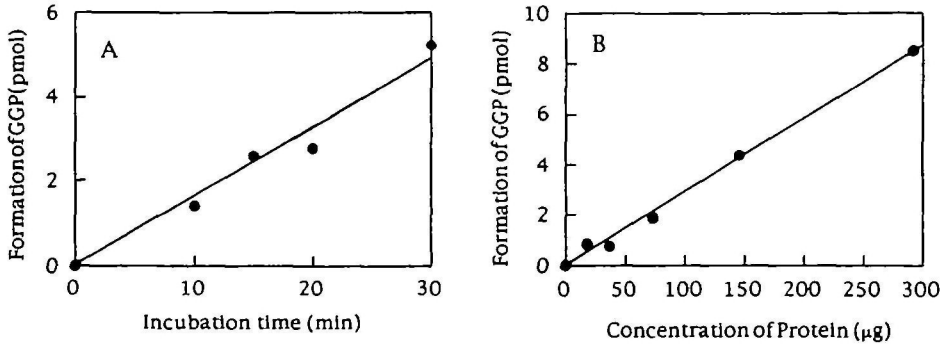

Fig. 2. **Activity of GGOH kinase as a function of incubation time (A) and the amount of 100,000×*g* precipitate (B).** A: The reaction mixture contained 253 nM [1-^{14}C]GGOH (1.92 GBq/mmol), 10 mM ATP, 20 mM $MgCl_2$, 16 mM CHAPS, and 40 μg of 100,000×*g* precipitate. The reaction was carried out for the time indicated. B: The reaction mixture contained 220 nM [1-^{14}C]GGOH (1.92 GBq/mmol), 10 mM ATP, and an indicated amount of 100,000×*g* precipitate. The reaction was carried out for 10 min. The activity was determined as described under "EXPERIMENTAL PROCEDURES."

sodium deoxycholate, sodium cholate, and octylglucoside, have been unsuccessful so far. Therefore we used a suspension of the 100,000×*g* precipitate to characterize the GGOH kinase.

General Properties of the GGOH Kinase—The time course of the GGOH kinase reaction was linear for at least 30 min (Fig. 2A). The formation of product was also proportional to the amount of membrane protein (Fig. 2B). The pH-rate profile is a convex curve with a maximum between 6.5 and 8.5 (data not shown).

Phosphate-Donor Specificity of GGOH Kinase—To investigate the phosphate donor specificity of GGOH kinase, four nucleotides (ATP, GTP, CTP, and UTP) and three isoprenyl diphosphates (GGPP, FPP, and IPP) were tested as substrates. As shown in Table II, all four nucleotides served as phosphate donors, and ATP was more active than the other nucleotides examined. The Michaelis constant for ATP was determined at 55°C in 0.22 μM [1-^{14}C](all-*E*)-GGOH, 20 mM $MgCl_2$, 32 mM CHAPS, and 0.1 M Tris-HCl, pH 7.0. Under these conditions, K_m^{ATP} was 0.65 mM. Incubation without a nucleotide did not yield GGP. The three isoprenyl diphosphates were inactive.

Polyprenol Specificity of GGOH Kinase—Radiolabeled polyprenyl diphosphates, [1-^{14}C](all-*E*)-GGPP, [1,5,9-^{14}C]-(all-*E*)-hexaprenyl diphosphate, [1,5,9,13,17-^{14}C](all-*E*)-octaprenyl diphosphate, and [1,5,9,13,17,21,25,29-^{14}C]-(2*Z*,6*Z*,10*Z*,14*Z*,18*Z*,22*Z*,26*Z*,30*Z*,34*E*,38*E*)-undecaprenyl diphosphate, were enzymatically synthesized with GGPP synthase from *S. acidocaldarius*, hexaprenyl diphosphate synthase from *M. luteus* B-P26, solanesyl diphosphate synthase from *M. luteus*, and undecaprenyl diphosphate synthase from *Bacillus subtilis*, respectively. The diphosphate moieties were hydrolyzed by acid phosphatase treatment, and the resulting polyprenols were purified by reversed-phase thin-layer chromatography to remove the polyprenols derived from the primers of the prenyltransferase reactions. [1-^{3}H]Geraniol and [1-^{3}H]-(all-*E*)-farnesol were obtained from commercially available substrates. As shown in Table III, (all-*E*)-GGOH was the best substrate. The apparent K_m value for GGOH when assayed at 10 mM ATP, 20 mM $MgCl_2$, 32 mM CHAPS, and 0.1 mM Tris-HCl, pH 7.0, was 27 nM. (all-*E*)-Farne-

TABLE II. **Effect of phosphate donor substrate on GGOH kinase activity.** Reaction mixtures contained 253 nM [1-^{14}C]GGOH (1.92 GBq/mmol), 20 mM $MgCl_2$, 16 mM CHAPS, 40 μg of 100,000×*g* precipitate, and the indicated amount of the phosphate donor substrate. After a 1-h incubation at 55°C, the amount of [1-^{14}C]-GGP formed was measured as described under "EXPERIMENTAL PROCEDURES."

Addition to reaction mixture	GGOH kinase activity (pmol/mg protein)	Relative activity (%)
None	0	0
ATP (10 mM)	163	100
GTP (10 mM)	141	86.5
CTP (10 mM)	103	63.2
UTP (10 mM)	123	75.5
GGPP (0.2 mM)	0	0
FPP (0.2 mM)	0	0
IPP (0.2 mM)	0	0

TABLE III. **Polyprenol specificity of GGOH kinase.** Reaction mixtures contained 10 mM ATP, 20 mM $MgCl_2$, 16 mM CHAPS, 100,000×*g* precipitate, and the indicated radioactive polyprenol. After a 1-h incubation at 55°C, the amount of polyprenyl phosphate formed was measured as described under "EXPERIMENTAL PROCEDURES."

Addition to reaction mixture	Relative activity (%)
Geraniol	0
(all-*E*)-Farnesol	6.81
(all-*E*)-GGOH	100
(all-*E*)-Hexaprenol	37.5
(all-*E*)-Octaprenol	23.5
(2*Z*,6*Z*,10*Z*,14*Z*,18*Z*,22*Z*,26*Z*, 30*Z*,34*E*,38*E*)-Undecaprenol	0

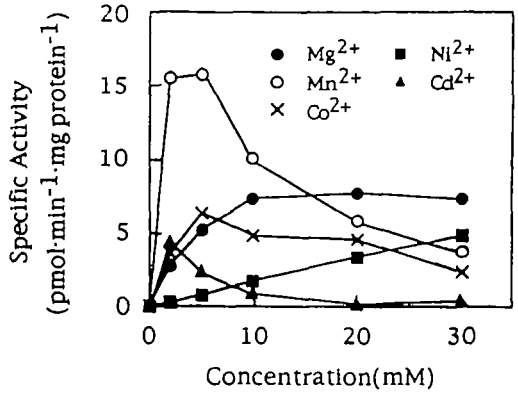

Fig. 3. **Divalent cation dependence of GGOH kinase.** Each reaction mixture contained the standard buffer components plus 0.22 μM [1-^{14}C]GGOH (1.92 GBq/mmol), 10 mM ATP, 16 mM CHAPS, 63 μg of 100,000×*g* precipitate, and the indicated amount of a divalent metal ion. The samples were incubated for 1 h at 55°C and then the activity was analyzed by thin-layer chromatography as described in "EXPERIMENTAL PROCEDURES."

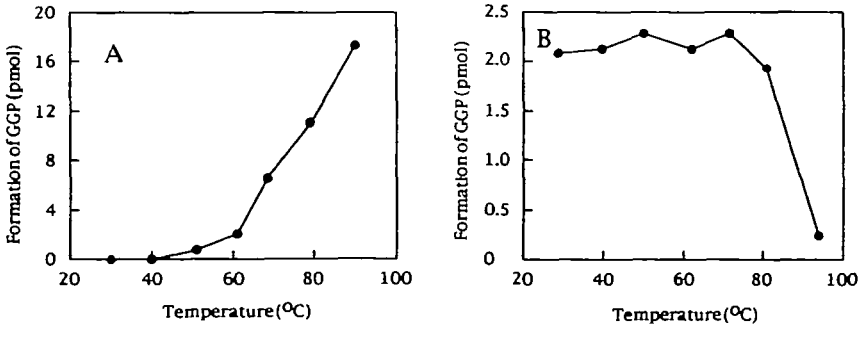

Fig. 4. **Thermostability of GGOH kinase.** A: Each reaction mixture contained 0.23 μM [1-^{14}C]GGOH (1.92 GBq/mmol), 10 mM ATP, 20 mM $MgCl_2$, 16 mM CHAPS, and 31.5 μg of 100,000×*g* precipitate. The samples were incubated for 10 min at the indicated temperature, and then the activity was analyzed by thin-layer chromatography as described in "EXPERIMENTAL PROCEDURES." B: The 100,000×*g* precipitate was heated at an indicated temperature for 1 h without substrates and then the remaining activity in 0.1 M Tris-HCl buffer, pH 7.0, containing 0.21 μM [1-^{14}C]GGOH (1.92 GBq/mmol), 10 mM ATP, 20 mM $MgCl_2$, 16 mM CHAPS, and 146 μg of 100,000×*g* precipitate was determined as described under "EXPERIMENTAL PROCEDURES."

sol, (all-*E*)-hexaprenol, and (all-*E*)-octaprenol also served as substrates, exhibiting approximately 6.8, 37, and 23% of the activity of GGOH under similar conditions, respectively. However, geraniol and (2*Z*,6*Z*,10*Z*,14*Z*,18*Z*,22*Z*,26*Z*,30*Z*,34*E*,38*E*)-undecaprenol were not acceptable as substrates.

Effect of Divalent Cations on the Activity of GGOH Kinase—We examined the effect of a broad spectrum of divalent cations on GGOH kinase. Nine different cations (Zn^{2+}, Ca^{2+}, Co^{2+}, Mg^{2+}, Mn^{2+}, Cd^{2+}, Ni^{2+}, Cu^{2+}, Hg^{2+}) were added to the reaction mixture at a final concentration of 5 mM. The GGOH kinase showed no activity in the absence of cations. Zn^{2+}, Co^{2+}, Mg^{2+}, Mn^{2+}, and Ni^{2+} were found to be effective in promoting phosphorylation of GGOH. The only product obtained from the reactions using the divalent metal ions described above was GGP. The diphosphate derivative was not formed (data not shown). The formation of GGP was completely abolished by the addition of EDTA. Figure 3 shows the effect of various amounts of Mn^{2+}, Co^{2+}, Mg^{2+}, Ni^{2+}, and Cd^{2+} on the activity of GGOH kinase. Mn^{2+} showed the greatest activation at 5 mM, and the activation was dramatically decreased as the concentration was increased. In the case of Mg^{2+}, the enzyme activity increased and then reached a plateau. Although the species of divalent metal ion that activate the phosphorylation of GGOH are almost the same as those in the cases of other isoprenoid alcohol kinases (*11, 12*), the efficiencies of the divalent metal ions are different from those seen with other isoprenoid alcohol kinases.

Thermostability of GGOH Kinase—The Archaebacterium *S. acidocaldarius* can be grown at temperatures of up to 85°C. It has been reported that almost all enzymes of this bacterium are extremely thermostable. We analyzed the thermostability of GGOH kinase using two systems. Activity of GGOH kinase was assayed over the temperature range of 30-90°C for 10 min (Fig. 4A). No activity was observed below 40°C. As the temperature was increased, the activity of GGP increased. In a second system, the enzyme solution was heated without substrate at the indicated temperature for 1 h, and then assayed for remaining activity at 55°C for 10 min (Fig. 4B). Most of the activity remained even after heating at 80°C. The activity was dramatically decreased at 94°C. These data confirm the extraordinary thermostability of the kinase.

DISCUSSION

We have observed that the cell lysate of the extremely acidothermophilic Archaebacterium *S. acidocaldarius* has the ability to convert GGOH to GGPP. This observation is consistent with that in a GGOH feeding experiment by Poulter *et al.* (*6*). Ultracentrifugation easily separated the phosphorylation enzymes into two components, GGOH kinase and GGP kinase, which exist in the membrane fraction and cytosolic fraction, respectively. The subcellular distribution of the two enzymes is consistent with the hydrophobicity of the corresponding isoprenoid substrates.

In eukaryotes, GGPP is a precursor of geranylgeranylated proteins, which participate in many cellular events, including the control of cytoskeleton-membrane interaction and the regulation of vesicular fusion during secretion and endocytosis. Recently, it was reported that geranylgeraniol was incorporated into geranylgeranylated protein (*13*) and that the same prenol induced apoptosis in HL-60 cells (*14*). These data suggest the existence and important roles of GGOH kinase and GGP kinase. The kinases found from *S. acidocaldarius* might be ancestors of such eukaryotic enzymes.

So far, three isoprenol kinases have been found in organisms other than Archaebacteria. Dolichol kinase was found in eukaryotes, and it phosphorylates dolichol, which is a polyprenol with a saturation of the α-terminal double bond, to give its phosphate, which serves as a cofactor in the formation of *N*-linked glycoproteins (*15*). Undecaprenol kinase, found in bacteria, phosphorylates undecaprenol to give its phosphate, which serves as the glycosyl carrier lipid in prokaryotic peptideglycan biosynthesis (*16, 17*). Recently, a new isoprenol kinase, farnesol kinase, was found in the microalga *Botryococcus brauni* (*7*). The specificities of these kinases towards isoprenoid alcohols of different chain lengths have been reported by several groups (*7, 16, 18-20*). Dolichol kinase phosphorylates a wide range of dolichols differing in the number of isoprene units. The maximum activity was observed when C_{95} dolichol was used, and the activity decreased as the chain length became shorter. Fully unsaturated polyprenols were also phosphorylated by the kinase. Dolichols and polyprenols with similar chain lengths showed similar reactivities (*19*). Undecaprenol kinase also catalyzed the phosphorylation of (all-*E*)-nonaprenol and dolichol with 85 and 13% of the activity of undecaprenol, respectively. However, C_{20} polyprenols, (all-*E*)-GGOH and (2*Z*,6*E*,10*E*)-GGOH, were very poor substrates (*16*). Farnesol kinase only catalyzed phosphorylation of (all-*E*)-farnesol. Significant phosphorylation was not detected when farnesol was replaced with geraniol, (all-*E*)-GGOH, *Z*,*E*-mixed polyprenols, or dolichols (*7*). Before our experiment on the polyprenol specificity, we suspected that the phosphorylation of GGOH might be catalyzed by dolichol kinase in *S. acidocaldarius*. However, we showed that GGOH was the best substrate for the kinase and that the activity of undecaprenol was below 0.2% of that of GGOH. This finding clearly indicates that the phosphorylation of GGOH is due to a specific kinase.

In archaebacteria, except for some methanobacteria, glycoproteins in the cell membrane work to maintain the shape of cells as in the case of peptideglycans in eubacteria (*21*). The cell wall from *S. acidocaldarius* contains two glycoproteins of molecular weight 40,000 and 100,000 as major subunits, with glucose and mannose as major carbohydrate components (*22*). The biosynthesis of archaebacterial glycoproteins was only reported for halophiles. The

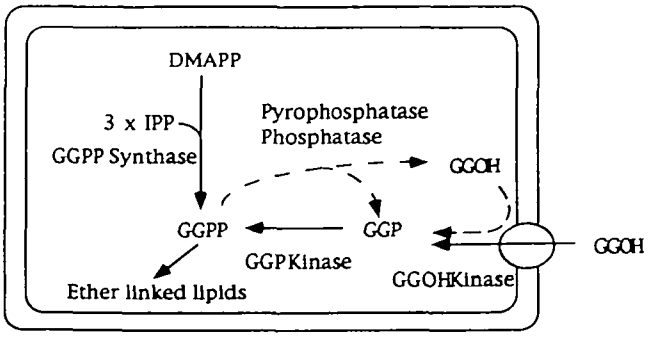

Fig. 5. **Possible role of GGOH kinase.**

dolichyl compound whose chain length is C_{60} acts as a sugar carrier (*23*). Therefore, its membrane fraction must contain dolichol kinase. The dolichol kinase previously reported can also catalyze phosphorylation of unsaturated polyprenols with chain lengths similar to those of dolichols. However, we could not observe undecaprenol kinase activity. Our assay conditions may not be suitable for detecting dolichol kinase.

GGOH kinase utilized all nucleotides tested as phosphoryl donors. This kinase is quite different from the previously reported isoprenoid alcohol kinases, each of which requires a specific nucleotide (*7, 24-28*). There is a possibility that the 100,000×*g* precipitate of *S. acidocaldarius* may contain nucleoside diphosphate kinase, which utilizes any nucleoside triphosphate as a phosphoryl donor to produce a nucleoside triphosphate that is really accepted by GGOH kinase. However, almost all of the previously reported nucleoside diphosphate kinases were cytosolic (*29-31*). Since undecaprenol kinase in bacteria utilizes ATP as a phosphate donor, we measured the amount of ATP formation using the luciferase reaction when the 100,000×*g* precipitate was incubated with nucleoside triphosphates other than ATP. No significant formation of ATP was detected (data not shown). Therefore the four nucleoside triphosphates seem to be directly utilized by GGOH kinase.

The discovery of a GGOH kinase and a GGP kinase in *S. acidocaldarius* indicates that GGOH formed in the cell or incorporated from the environment is actually utilized as a precursor of ether-linked lipids (Fig. 5). What is the real role of phosphorylation of GGOH? It may be concerned with a salvage pathway or regulation of ether-linked lipid biosynthesis *via* a phosphorylation-dephosphorylation cycle of GGOH. Two types of pyrophosphatases in *S. acidocaldarius* were identified and characterized. One is a membrane-bound enzyme (*32*) and the other is a cytosolic enzyme (*33*). Meyer and Schafer postulated that a possible function of the pyrophosphatase was the hydrolysis of dolichol diphosphate in connection with glycosylation reactions of membrane proteins. However the substrate specificity is not known in either case. Moreover, we observed phosphatase activity that produced GGOH from its diphosphate derivative (data not shown). Therefore, one of them may catalyze the hydrolysis of GGPP. Recently, it has been reported that farnesol plays an important role in the regulation of sterol biosynthesis (*34*). It seems important to elucidate the role of the GGOH phosphorylation.

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