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Biosynthesis of Archaeal Membrane Lipids: Digeranylgeranylglycerophospholipid Reductase of the Thermoacidophilic Archaeon *Thermoplasma acidophilum*

Yuji Nishimura and Tadashi Eguchi*
Department of Chemistry & Materials Science, Tokyo Institute of Technology, O-okayama, Meguro-ku, Tokyo 152-8551

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The basic core structure of archaeal membrane lipids is 2,3-di-*O*-phytanyl-*sn*-glyceryl phosphate (archaetidic acid), which is formed by the reduction of 2,3-di-*O*-geranylgeranylglyceryl phosphate. The reductase activity for the key enzyme in membrane lipid biosynthesis, 2,3-digeranylgeranylglycerophospholipid reductase, was detected in a cell free extract of the thermoacidophilic archaeon *Thermoplasma acidophilum*. The reduction activity was found in the membrane fraction, and FAD and NADH were required for the activity. The reductase was purified from a cell free extract by ultracentrifugation and four chromatographic steps. The purified enzyme showed a single band at ca. 45 kDa on SDS-PAGE, and catalyzed the formation of archaetidic acid from 2,3-di-*O*-geranylgeranylglyceryl phosphate. Furthermore, the enzyme also catalyzed the reduction of 2,3-di-*O*-geranylgeranylglyceryl phosphate analogues such as 2,3-di-*O*-phytyl-*sn*-glyceryl phosphate, 3-*O*-(2,3-di-*O*-phytyl-*sn*-glycero-phospho)-*sn*-glycerol and 2,3-di-*O*-phytyl-*sn*-glycero-phosphoethanolamine. The *N*-terminal 20 amino acid sequence of the purified enzyme was determined and was found to be identical to the sequence encoded by the Ta0516m gene of the *T. acidophilum* genome. The present study clearly demonstrates that 2,3-digeranylgeranylglycerophospholipid reductase is a membrane associated protein and that the hydrogenation of each double bond of 2,3-digeranylgeranylglycerophospholipids is catalyzed by a single enzyme.

Key words: archaea, biosynthesis, membrane lipid, reductase, *Thermoplasma acidophilum*.

Abbreviations: GGPP, geranylgeranyl diphosphate; DGGGP, 2,3-di-*O*-geranylgeranylglyceryl phosphate; d_{12}-DGGGP, 2,3-di-*O*-[16,17-d_6]geranylgeranyl-*sn*-glyceryl phosphate; DPHGP, 2,3-di-*O*-phytyl-*sn*-glyceryl phosphate; DPHGPG, 3-*O*-(2,3-di-*O*-phytyl-*sn*-glycero-phospho)-*sn*-glycerol; DPHGPE, 2,3-di-*O*-phytyl-*sn*-glycero-phosphoethanolamine; FAB MS, fast atom bombardment mass spectrometry; PMSF, phenylmethylsulfonyl fluoride.

Archaea, which have been attracting considerable attention for both their biochemical and evolutionary aspects, are distinct from bacteria and eukarya, and are now classified as a third independent domain (*1*). One of the most significant differences between archaea and other organisms lies in the structure of core membrane lipids. Archaeal cell membrane lipids are composed of saturated isoprenoid chains linked to a glycerol molecule at the *sn*-2 and -3 positions by ether linkage (*2*). Fatty acid components are completely absent. Thus, the core lipid structure is considered to be the most crucial feature for the classification of archaea apart from other organisms.

The basic core structure of the archaeal membrane is 2,3-di-*O*-phytanyl-*sn*-glycerol (*3*, *4*). The isoprenoid hydrocarbon chains of the lipid molecules are frequently joined at the terminal position to form macrocyclic rings with as many as 36- (macrocyclic archaeol) or 72- (caldarchaeol) members in some methanogenic and thermophilic archaea (*5*, *6*). The biosynthesis of the basic archaeal membrane lipids is now considered to proceed as shown in Fig. 1. Nishihara *et al*. reported that the glycerol moiety is generated from dihydroxyacetone phosphate by glyceryl-*sn*-1-phosphate dehydrogenase of *Methanothermobacter thermoautotrophicus*. This enzyme class occurs only in archaea (*7*, *8*). The isoprenoid chains are mainly derived from geranylgeranyl diphosphate, which is biosynthesized *via* the mevalonate pathway (*9*–*11*). Zhang and Poulter showed that the formation of ether linkages between glyceryl-*sn*-1-phosphate and the isoprenoid chains of *M. thermoautotrophicus* are catalyzed by two independent prenyltransferases. In a first step, 3-*O*-geranylgeranylglyceryl phosphate synthase catalyzes the transfer of the geranylgeranyl group from geranylgeranyl diphosphate to the C3 position of glyceryl-*sn*-1-phosphate. This enzyme was first purified from *M. thermautotrophicus* by Poulter *et al*., and orthologous proteins have been described from *M. thermautotrophicus* and *T. acidophilum* (*9*, *12*, *13*). The second step enzyme, digeranylgeranylglyceryl phosphate synthase, which converts 3-*O*-geranylgeranylglyceryl phosphate into 2,3-di-*O*-geranylgeranylglyceryl phosphate, this enzyme was cloned from *Sulfolobus solfataricus* by Hemmi *et al*. (*14*). Some enzymes involved in the conversion of the polar head group have also been found in recent years (*15*, *16*). However, the key

*To whom correspondence should be addressed. Tel/Fax: +81-3-5734-2631, E-mail: eguchi@cms.titech.ac.jp3

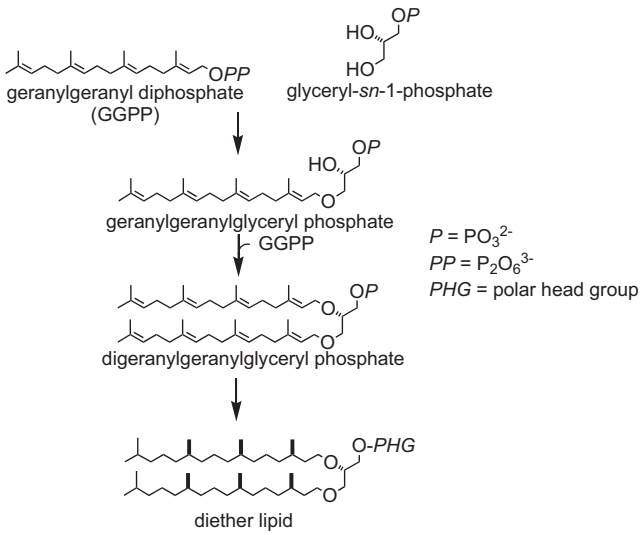

Fig. 1. **Biosynthetic pathway of archaeal membrane lipids.**

biosynthetic step, the reduction of geranylgeranyl chains, remained to be clarified.

To obtain insight into this reduction reaction, we studied the enzymatic reduction of digeranylgeranylglyceryl phosphate, a possible biosynthetic intermediate, using cell free extracts from several halophilic, methanogenic, and thermoacidophilic archaea. Among the organisms tested here, the hydrogenation activity was detected only in the thermoacidophilic archaeon *Thermoplasma acidophilum*, whose core lipids are composed of archaeol and caldarchaeol containing a 0–8 cyclopentane ring (*17*). The enzyme was purified to homogeneity, and the coding gene was functionally assigned to encode digeranylgeranylgycerophospholipid reductase.

MATERIALS AND METHODS

Generals—^{2}H NMR spectra were recorded on an LA-400 spectrometer (JEOL). Fast atom bombardment mass spectrometry (FAB MS) was performed with an MS station JMS-700 mass spectrometry system (JEOL). Chloroform was used as a solvent for ^{2}H NMR spectroscopy. ^{2}H chemical shifts were reported as δ values based on the natural abundance signal of C^2HCl_3 (δ_{2H} = 7.26) as a reference. Column chromatography was carried out with Kiesegel 60 (70–230 mesh, Merck). A Pharmacia fast protein liquid chromatograph was used for protein purification. Proteins were purified at 4°C and detected at 280 nm. Ultrafiltration was performed by centrifugation with M_r 10,000 cut-off tubing made of polyether sulfone membrane (VIVASIENCE). Fractions containing the reductase activity were pooled and analyzed by 12.5% SDS-PAGE. Protein mass was estimated by SDS-PAGE using Perfect Protein Markers (TAKARA). Protein bands were stained with a the Silver stain KANTO III kit. Protein concentrations were determined by the BCA method using bovine serum albumin as a standard.

Chemicals—2,3-Di-*O*-geranylgeranyl-*sn*-glyceryl phosphate (DGGGP), was synthesized in our laboratory (*9*). 2,3-Di-*O*-[16,17-d_6]geranylgeranyl-*sn*-glyceryl phosphate (d_{12}-DGGGP), 2,3-di-*O*-phytyl-*sn*-glyceryl phosphate (DPHGP), 2,3-di-*O*-phytyl-*sn*-glycero-phosphoethanolamine (DPHPE) and 3-*O*-(2,3-di-*O*-phytyl-*sn*-glycerophospho)-*sn*-glycerol (DPHGPG) were prepared essentially according to the method previously reported (*18*).

Culture Conditions—*T. acidophilum* JCM 9062 was obtained from the Japan Collection of Microorganisms (RIKEN). The medium ingredients, $(NH_4)_2SO_4$ 0.2 g; KH_2PO_4 3.0 g; $MgSO_4{\cdot}7H_2O$ 0.5 g; $CaCl_2{\cdot}2H_2O$ 0.25 g; Bacto yeast extract (Difico) 1.0 g; glucose 10.0 g; distilled water 1.0 liters were mixed and the mixture was adjusted to pH 2.0 by adding H_2SO_4 (*19*). The medium was sterilized by filtration, and then was inoculated. Cultivation was carried out for 48–72 h at 60°C, and the cells were collected by centrifugation (6,500–9,000 rpm, 30 min). The wet cells were washed with distilled water adjusted to pH 2 with H_2SO_4, and then with 50 mM phosphate buffer (pH 6.5).

Preparation of a Cell Free Extract—*T. acidophilum* cells (1 g of wet cells) were suspended in 10 ml of buffer A (50 mM potassium phosphate, 5% glycerol, pH 6.5) and 20 μl of 50 mM PMSF and disrupted by sonication (30 s × 8). The cell free extract was obtained by centrifugation at 10,000 × *g* for 20 min. The membrane fraction was obtained by ultracentrifugation at 170,000 × *g* for 2.5 h at 4°C. The membrane fraction was suspended in buffer B (buffer A + 0.3 M KCl).

Assay of the Crude Extract—An assay mixture containing 0.3 mM d_{12}-DGGGP, 5 mM NADH, 0.1 mM FAD, and 1.5% Triton X-100 was incubated with 10 ml of a suspension of the membrane fraction at 37°C for 12 h. The reaction mixture was extracted with $CHCl_3$, and the $CHCl_3$ extracts were washed with saline and concentrated by evaporation. A solution of diazomethane/ether (3 ml, 0.4 M) was added to the residue in ether (0.5 ml) at 0°C, and the mixture was stirred for 1 h to give methyl esters of lipids. The solution was concentrated to dryness, and the residue was chromatographed over silica gel with hexane-ethyl acetate (2:1). The product was dissolved in $CHCl_3$ and analyzed by ^{2}H-NMR spectrometry. The FAB MS spectra of the products were measured with a matrix of *m*-nitrobenzyl alcohol in a positive mode.

Cofactor Requirement—A reaction mixture contained 0.3 mM d_{12}-DGGGP, 1.5% Triton X-100 in 2.5 ml of the membrane fraction. The reaction was started by the addition of 0.5 ml of 5 mM NAD(P)H (or 5 mM dithionite), 0.1 mM FAD/FMN, 5 mM EDTA. The reaction mixture was incubated at 37°C for 12 h, and then the mixture was extracted with $CHCl_3$. The $CHCl_3$ extracts were washed with saline and concentrated by evaporation. The residue was dissolved in $CHCl_3$ and analyzed by ^{2}H-NMR spectrometry.

Purification of Digeranylgeranylglycerophospholipid Reductase—Wet *T. acidophilum* cells (12 g, 20 liter culture) were suspended in 40 ml of buffer A (50 mM potassium phosphate, 5% glycerol, 1 mM PMSF, pH 6.5) and the cells were disrupted by sonication at 4°C. The homogenate was centrifugated for 20 min at 10,000 × *g*, and the supernatant was ultracentrifugated at 170,000 × *g* for 2.5 h at 4°C. The precipitate was suspended in 20 ml of buffer A, and then homogenized with a glass homogenizer at 4°C. To the homogenate, 3 ml of 10% Triton

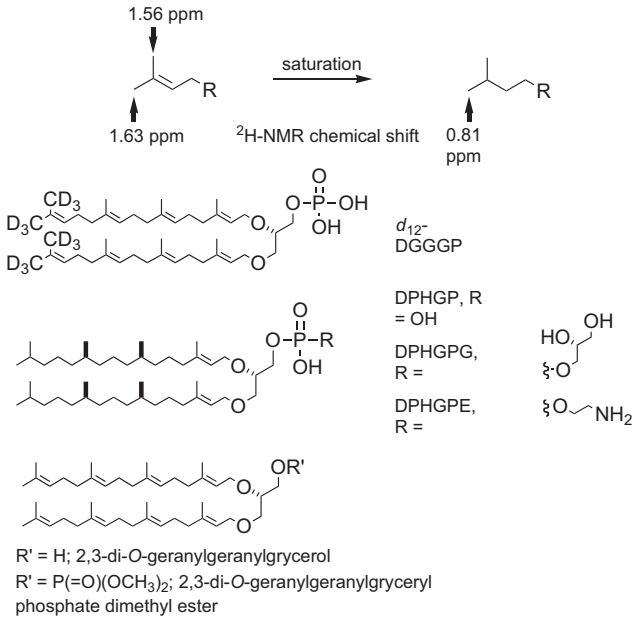

Fig. 2. **Assay method and structure of d_{12}-DGGGP.**

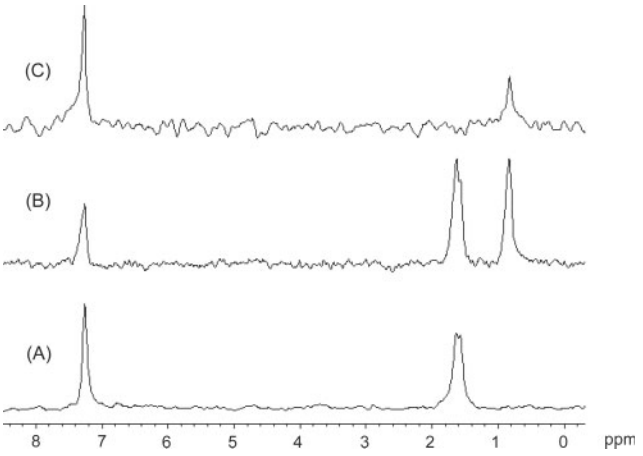

Fig. 3. **^{2}H NMR spectra (61 MHz, $CHCl_3$) of (A) authentic d_{12}-DGGGP dimethyl ester, (B) methyl ester of the enzyme reaction products of d_{12}-DGGGP and the membrane fraction, and (C) methyl ester of the enzyme reaction product of d_{12}-DGGGP and the purified reductase after 48 h at 50°C.**

X-100 and 200 μl of 10 mM FAD were added, and the mixture was incubated overnight at 4°C. The mixture was ultracentrifugated at 70,000 × *g* for 30 min at 4°C.

The supernatant was loaded onto a 1 × 21-cm DEAE Sepharose F.F. column (Amersham) equilibrated with 200 ml of the starting buffer (50 mM potassium phosphate, 0.1% Triton X-100, 5% glycerol, pH 6.5). The column was washed with 40 ml of the starting buffer and eluted with 50 mM KCl in starting buffer. The 50 mM KCl fractions were combined and concentrated, and the buffer was exchanged with buffer B (10 mM potassium phosphate, 5% glycerol, and 0.1% Triton X-100, pH 6.8) by ultrafiltration. The concentrated sample was loaded onto a 1 × 6-cm CHT

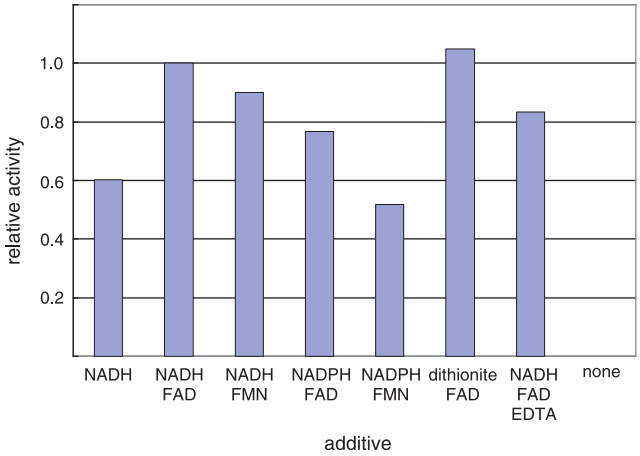

Fig. 4. **Effects of cofactors on the hydrogenation activity.** The conditions of the experiments were the same as described in "MATERIALS AND METHODS."

ceramic hydroxyapatite column (Bio-Rad) equilibrated with buffer B. The column was washed with 20 ml of buffer B and eluted with 120 ml of a 10–180 mM linear gradient of potassium phosphate at 0.2 ml/min. The active fractions were pooled and concentrated, and then the buffer of the mixture was exchanged with buffer C (25 mM histidine-HCl, 10% glycerol, and 0.1% Triton X-100, pH 6.2) by ultrafiltration. The concentrated sample was loaded onto a 0.7 × 15-cm PBE 94 column (Amersham) equilibrated with buffer C for chromatofocusing. The column was eluted with 100 ml of elution buffer (Polybuffer 74-HCl, dilution factor = 1:9, Amersham, 10% glycerol, and 0.1% Triton X-100, pH 4.5). The enzyme eluted at pH 5.1–5.0. The active fractions were pooled and concentrated, and then the buffer of the mixture was exchanged with buffer D (25 mM Tris-HCl, 10% glycerol, and 0.05% Triton X-100, pH 7.5) by ultrafiltration. The concentrated sample was loaded onto a Mono Q HR 5/5 column (Pharmacia) equilibrated with 20 ml of buffer D. The column was washed with buffer D and 100 mM NaCl in buffer D and eluted with a 15-ml linear gradient of 100–300 mM NaCl at 0.5 ml/min to give purified enzyme fractions. At this stage, we found that the reductase was stable even at higher temperature, which correlates with the origin of the enzyme. Therefore, the following enzyme reactions were conducted at 50°C.

Assay of the Purified 2,3-Digeranylgeranylglycerophospholipid Reductase—An assay mixture (500 μl) containing the purified enzyme (60 μg), 100 mM potassium phosphate, 0.2 mM FAD, 0.7 mM d_{12}-DGGGP, non-labeled DGGGP, DPHGP, DPHGPG, or DPHGPE, 0.5% Triton X-100, 20% glycerol, and 20 mM dithionite was incubated at 50°C for 48 h. The reaction was terminated by the addition of 2 M hydrochloric acid, and the reaction mixture was extracted with $CHCl_3$. The $CHCl_3$ extracts were washed with saline and concentrated. In the case of DPHGPE, the residue was treated with 0.5 ml of acetic anhydride/$CHCl_3$ (1:10) overnight before treatment with diazomethane solution, and then the mixture was concentrated. The residue was suspended in 0.5 ml of ether, a solution of diazomethane/ether (1 ml, 0.4 M) was added at 0°C, and the mixture was stirred for 1 h to give methyl esters of

Table 1. **Purification of 2,3-digeranylgeranylglycerophospholipid reductase from *T. acidophium*.**

Steps	Protein (mg)	Activity (nmol/min)	Recovery (%)	Specific activity[a] (nmol/min mg)	Purification (fold)
Cell free extract	3,360	13	100	0.004	1
Membrane fraction	1,160	9.3	69	0.008	2
DEAE Sepharose F.F	61	9.0	67	0.15	37
CHT (hydroxyapatite)	9.6	13	96	1.34	340
PBE94 (chromatofocusing)	0.90	9.0	67	9.96	2,500
MonoQ	0.15	1.7	13	11.5	2,900

Assays were performed in 50 mM phosphate buffer, pH 6.5, containing 0.7 mM DGGGP, 20 mM dithionite at 50°C. [a]Activity is expressed as reduction per one double bond.

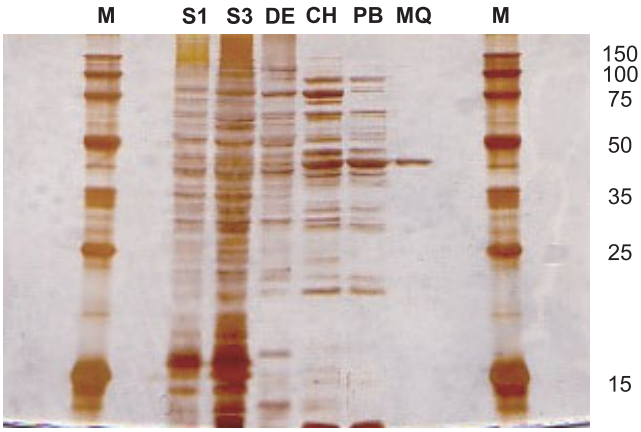

Fig. 5. **SDS-PAGE analysis of digeranylgeranylglycerophospholipid reductase.** 12.5% SDS-PAGE of the proteins at each step in the purification. S1, crude extract; S3, membrane fraction; DE, DEAE Sepharose F.F.; CH, CHT (hydroxyapatite); PB, PBE94 (chromatofocusing); MQ, MonoQ; M, protein standards.

lipids. The mixture was concentrated to dryness. The residue was chromatographed over silica gel with hexane-ethyl acetate, and analyzed by ^{2}H-NMR and/or FAB-MS.

Determination of the N-Terminal Amino Acid Sequence of 2,3-Digeranylgeranylglycerophospholipid Reductase—The N-terminal sequence of the purified enzyme was determined on an Applied Biosystems Model 492 sequencer. The putative amino acid sequence for 2,3-digeranylgeranylglycerophospholipid reductase was searched in DDBJ (DNA database of Japan; http://www.ddbj.nig.ac.jp/) and aligned using the ClustalW program. A phylogenetic tree was constructed using the neighbor-joining method. All parameters of the program were at the default settings.

RESULTS

Cell free enzyme assay. In order to detect reductase activity in a cell free extract of archaeal cells, we developed an assay method using the deuterated substrate, 2,3-di-*O*-[16,17-d_6]geranylgeranyl-*sn*-glyceryl phosphate (d_{12}-DGGGP), and analyzed the reaction mixture by ^{2}H NMR spectroscopy. When the reduction reaction proceeds, vinyl methyl groups are converted into aliphatic methyl groups and the signals of the vinyl and deuterated methyl groups are expected to shift to a higher field region in the ^{2}H NMR spectrum. Actually, vinyl methyl signals of the substrate d_{12}-DGGGP were observed at 1.56 and 1.63 ppm, and the aliphatic methyl signals of the product appeared at 0.81 ppm in ^{2}H NMR (Fig. 2). Thus, it is anticipated that the enzymatic reduction of double bonds is easily detected by this method. In preliminary experiments, we studied the enzymatic reduction of d_{12}-DGGGP using cell free extracts from several archaea, including *Haloarcula japonica*, *Methanothermobacter thermautotrophicus*, *Methanocaldococcus jannaschii*, and *T. acidophilum*, but we detected the reduction activity only in cell free extracts of the thermoacidophilic archaeon, *T. acidophilum*.

A cell free extract of *T. acidophilum* was prepared by sonication, followed by centrifugation at 10,000 × *g*. Incubation of the cell free extract with d_{12}-DGGGP and NADH as a reductant in the presence of Triton X-100 as a detergent at 37°C for 12 h, extraction of the enzymatic mixture with chloroform, and derivatization into phosphoric acid methyl ester with diazomethane resulted in the detection of a new signal at 0.81 ppm by ^{2}H-NMR spectroscopy as shown in Fig. 3B. No signal was detected in a control experiment without the cell free homogenate or NADH, or with a boiled cell free homogenate.

The cell free extract was ultracentrifuged at 170,000 × *g* for 2.5 h at 4°C to separate the cytosolic supernatant and the membrane fraction. When incubated with d_{12}-DGGGP and NADH for 12 h at 37°C, the membrane fraction showed significant enzymatic activity, while the cytosolic fraction did not. These results suggest that the reduction reaction is catalyzed by a membrane associated protein.

Cofactor Requirements—The cofactor requirements of the reductase activity were elucidated using the above described membrane fraction, and the results of this study are shown in Fig. 4. The enzyme reaction proceeded effectively in the presence of NADH. When NADH was omitted, the activity significantly decreased. Furthermore, the addition of FAD increased the enzyme activity. When dithionite ion was added instead of NADH as a reductant to the enzyme reaction, the reduction reaction also proceeded. The strong metal chelator EDTA had little effect on the enzyme activity. These results clearly indicate that this enzyme does not require a metal for its activity.

Purification of Digeranylgeranylglycerophospholipid Reductase and Analysis of the N*-Terminal Amino Acid Sequence*—Digeranylgeranylglycerophospholipid reductase from *T. acidophilum* was purified to an electrophoretically homogeneous state as described in "MATERIALS AND METHODS." The activity guided purification procedure is summarized in Table 1. SDS-PAGE of the purified enzyme yielded a single band at ca. 45 kDa as shown

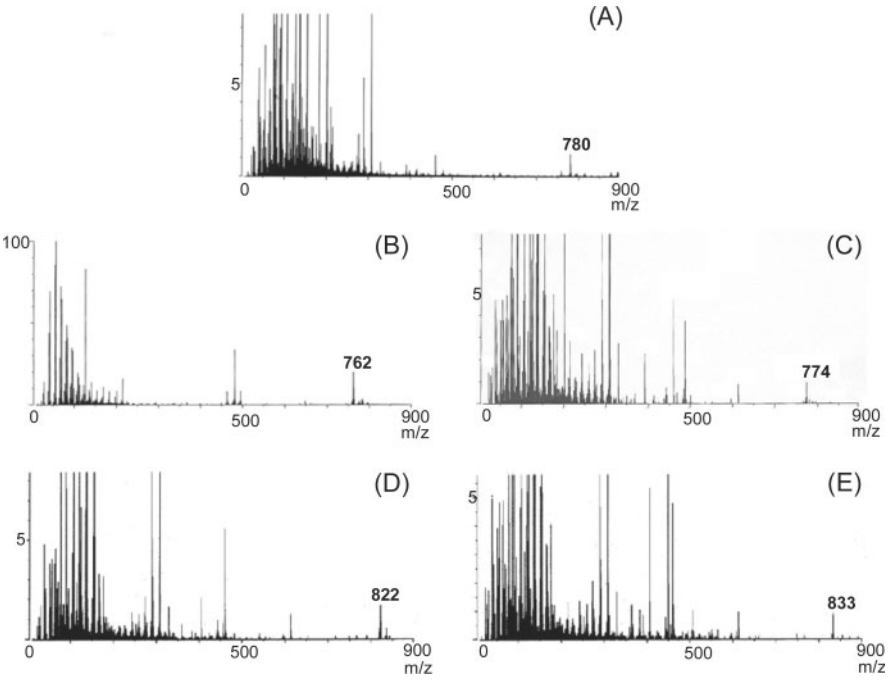

Fig. 6. **FAB-MS spectra of (A) authentic d_{12}-DGGGP dimethyl ester, (B) methyl ester of the enzyme reaction product of the purified reductase and non-labeled DGGGP, and (C) methyl ester of the enzyme reaction product of the purified reductase and d_{12}-DGGGP, (D) methyl ester of the enzyme reaction product of the purified reductase and DPHGPG, and (E) derivative of the enzyme reaction product of the purified reductase and DPHGPE.**

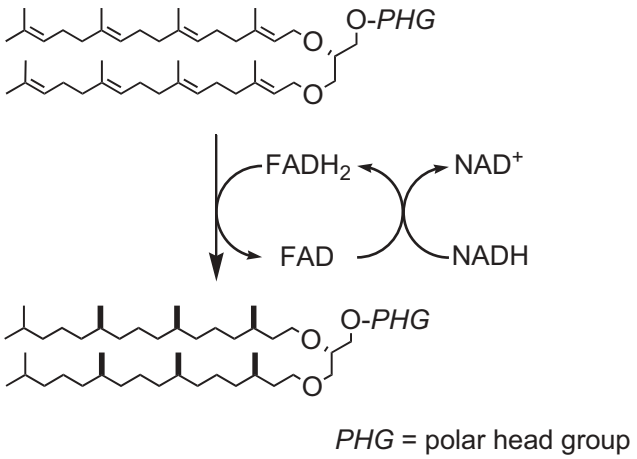

Fig. 7. **Putative hydrogenation reaction catalyzed by digeranylgeranylglycerophospholipid reductase.**

in Fig. 5. The N-terminal 20 amino acid sequence of the purified enzyme from *T. acidophilum* was determined as follows: M-E-T-Y-D-V-L-V-V-G-G-G-P-G-G-S-T-A-A-R-. This N-terminal sequence is identical to the N-terminal sequence encoded by the Ta0516m gene of the *T. acidophilum* genome.

Assay for the Purified 2,3-Digeranylgeranylglycerophospholipid Reductase and Product Analysis—The enzymatic reduction of d_{12}-DGGGP was carried out with the purified enzyme in the presence of FAD and dithionite as described in "MATERIALS AND METHOD," and the reaction product was analyzed by ^{2}H NMR spectroscopy after derivatization of the substrate and the products into the respective methyl esters. The derivatized lipids were easily purifed by silica gel chromatography. The vinylic methyl signals at 1.56 and 1.63 ppm of the substrate disappeared and a new signal at 0.81 ppm was observed as shown in Fig. 3C, which indicates that at least the terminal double bonds of the substrate were completely reduced by the enzyme. The reaction product was also analyzed by FAB MS spectrometry. The pseudo molecular ion peak of the reaction product was observed at *m/z* 774 $[M+H]^+$ in the positive mode as shown in Fig. 6C. The molecular mass of this ion is 16+1 atom mass units higher than the substrate (Mw 757), which indicates that all of the double bonds in the substrate were completely reduced. Interestingly, however, the pseudo molecular ion peak of the substrate, d_{12}-DGGGP, was observed at *m/z* 780 as the $[M+Na]^+$ ion (Fig. 6A). Although the exact reason for these phenomena is not clear at the moment, the presence of the double bonds may affect the formation of the pseudo molecular ions of these compounds in FAB-MS spectroscopy.

We also carried out the enzymatic reduction of non-labeled 2,3-di-*O*-geranylgeranylglyceryl phosphate with the purified enzyme, and derivatized and isolated the product in the same manner as described above. The pseudo molecular ion peak of the reaction product was observed at *m/z* 762 $[M+H]^+$ as shown in Fig. 6B. The molecular ion corresponds to 16+1 atom mass units higher than the substrate (Mw 745). Furthermore, the reaction product was in good agreement with the chemically synthesized 2,3-di-*O*-phytanylglyceryl phosphate dimethyl ester in all respects including ^{1}H NMR, FAB MS, and chromatographic behavior on TLC (data not shown).

The enzymatic reduction of DGGGP analogues such as DPHGP, DPHGPG, and DPHGPE was also carried out, and the products were derivatized and analyzed in the same manner as described above. The pseudo molecular ion peak of the reaction product from DPHGP was observed at *m/z* 762 as in the case of non-labeled DGGGP. In the reactions with DPHGPG and DPHGPE, the pseudo molecular ion peaks were observed at *m/z* 822 and 833 as

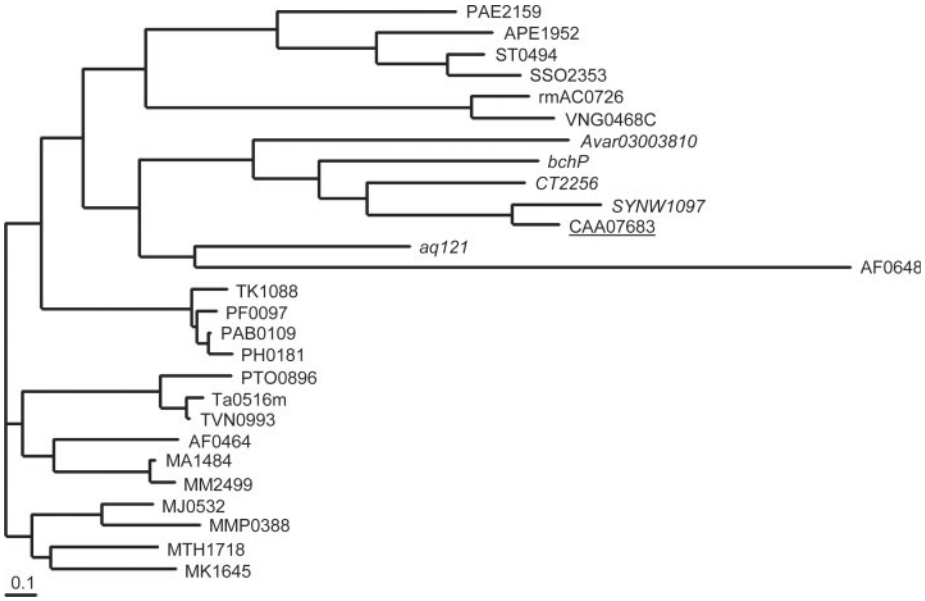

Fig. 8. **Homologues identified by BLAST searches.** The scale bar represents 0.1 substitutions per amino acid position. The abbreviation and accession number of each protein are as follows: bchP, geranylgeranyl bacteriochlorophyll reductase from *Rhodobacter sphaeroides* (CAB38733); Avar03003810, dehydrogenase from *Anabaena variabilis* strain ATCC 29413 (ZP_00159968); SYNW1097, geranylgeranyl hydrogenase from *Synechococcus* sp. strain WH 8102 (CAE07612); CT2256, geranylgeranyl hydrogenase from *Chlorobium tepidum* TLS (NP_663129); aq_121, hypothetical protein from *Aquifex aeolicus* VF5 (NP_213081); AF0648, menaquinone prenylreductase from *Archaeoglobus fulgidus*; Ta0516m, digeranylgeranylglycerophospholipid reductase from *Thermoplasma acidophilum* (NP_393992); TVN0993, a hypothetical protein from *Thermoplasma volcanium*, annotated as the geranylgeranyl reductase (NP_111512); MM2499, a hypothetical protein from *Methanosarcina mazei*, annotated as the geranylgeranyl reductase (NP_634523); MK1645, a hypothetical protein from *Methanopyrus kandleri*, annotated as the predicted dehydrogenase (NP_614928); PF0097, a hypothetical protein from *Pyrococcus furiosus*, annotated as the 43 kDa subunit bacteriochlorophyll synthase–like protein (NP_577826); TK1088, a hypothetical protein from *Thermococcus kodakarensis*, annotated as the geranylgeranyl hydrogenase (BAD85277); PAB0109, a hypothetical protein from *Pyrococcus abyssi*, annotated as the geranylgeranyl hydrogenase (CAB49088); PH0181, hypothetical protein from from *Pyrococcus horikoshii* (NP_142180); PTO0896, a hypothetical protein from *Picrophilus torridus*, annotated as the geranylgeranyl hydrogenase (AAT43481); AF0464, a hypothetical protein from *Archaeoglobus fulgidus*, annotated as bacteriochlorophyll synthase, 43 kDa subunit (NP_069300); MA1484, a hypothetical protein from *Methanosarcina acetivorans* C2A, annotated as the geranylgeranyl hydrogenase (NP_616418); MJ0532, a hypothetical protein from *Methanocaldococcus jannaschii*, annotated as bacteriochlorophyll synthase 43 kDa subunit (AAB98523); MTH1718, a hypothetical protein from *Methanothermobacter thermautotrophicus*, annotated as bacteriochlorophyll synthase 43 kDa subunit (AAB86190); MMP0388, a hypothetical protein from *Methanococcus maripaludis* S2, annotated as geranylgeranyl bacterial chlorophyll reductase (NP_987508); APE1952, a hypothetical protein from *Aeropyrum pernix* K1 (NP_148281); PAE2159, a hypothetical protein from *Pyrobaculum aerophilum* str. IM2 (NP_559809); ST0494, a hypothetical protein from *Sulfolobus tokodaii* str. 7 (NP_376378); SSO2353, a hypothetical protein from *Sulfolobus solfataricus* P2, annotated as geranylgeranyl hydrogenase (AAK42505); VNG0468C, a hypothetical protein from *Halobacterium* sp. NRC-1; rrnAC0726 a hypothetical protein from *Haloarcula marismortui* (AAV45719); CAA07683, geranylgeranyl reductase from *Nicotiana tabacum*. Plane, archaea; italic, bacteria; underlined, eukaryote.

shown in Fig. 6, D and E, respectively. Each of the observed molecular ion peaks corresponds to 4+1 atom mass units higher than that of the substrate (DPHGP Mw 757, DPHGPG Mw 817, DPHGPE Mw 828). Neither 2,3-di-*O*-geranylgeranylglycerol nor 2,3-di-*O*-geranylgeranylglyceryl phosphate dimethyl ester was recognized as a substrate (data not shown).

DISCUSSION

The enzymatic reduction of geranylgeranyl chains in the biosynthesis of archaeal membrane lipids is intriguing from the biosyntheti and physiological points of view. First, we recently reported that the presence of the terminal double bond of digeranylgeranyl groups is crucial for the formation of macrocyclic lipids, and that the reduction of the double bond at the terminus of a geranylgeranyl group appears to be a branching point leading either to diphytanyl glycerol lipid or macrocyclic lipids (*20*, *21*). Moreover, the data suggested that some archaea adapt their membrane lipid properties to the environment by adjusting the number of double bonds in the isoprenoid chains. Nichols *et al*. reported that the number of the double bonds in the membrane lipids of *Methanococcoides burtonii* changes in response to environmental temperature (*22*). In fact, Dannenmuller *et al*. reported that saturation of the isoprenoid chains significantly influences the biophysical properties of membrane lipids (*20*).

In the present study, we demonstrate the presence of a 2,3-di-*O*-geranylgeranylglyceryl phosphate reduction activity in cell free extracts of *T. acidophilum* in the presence of FAD and NADH. The progress of the reduction was successfully monitored by an assay method comprising the combinational use of deuterated 2,3-di-*O*-geranylgeranylglyceryl phosphate as a substrate and ^{2}H NMR analysis of the reaction products. Further, the reductase

Fig. 9. **Alignment of the amino acid sequences of digeranylgeranylglycerophospholipid reductase from *T. acidophilum* and homologues from seven archaeal and two bacterial species.** Abbreviations are the same as in the legend to Fig. 8. Conserved residues and similar residues are indicated by asterisks and dots, respectively.

was purified to an electrophoretically homogeneous state by ultracentrifugation and four chromatographic steps. Since the reduction of geranylgeranyl groups to phytanyl groups has been reported to take place by a *syn* addition of hydrogens to each double bond in incorporation experiments with mevalonolactone-d_9, the involvement of a single enzyme was suggested for the reduction reaction (*23*). In fact, the purified enzyme is capable of hydrogenating all of the double bonds in 2,3-di-*O*-geranylgeranylglyceryl phosphate. Therefore, the present study clearly confirms that a single enzyme catalyzes the reduction of 2,3-digeranylgeranylglycerophospholipid to 2,3-diphytanylglycerophospholipid.

Furthermore, the enzyme recognizes 2,3-digeranylgeranylglyceryl phosphate derivatives having phosphoethanolamine and phosphoglycerol as polar groups that are often found naturally. These results demonstrate that the enzyme, namely 2,3-digeranylgeranylglycerophospholipid reductase, catalyzes the reduction of unsaturated archaeols into saturated archaeols at various stages in the biosynthesis of archaeal membrane lipids. Unfortunately, the details of the enzyme kinetics could not be established because Triton X-100 interfered with the quantitative recovery of the lipidic products of the reaction.

The results described above indicate that the reductase is a membrane associated protein. It is known that enzymes involved in the biosynthesis of *sn*-3-*O*-geranylgeranylglyceryl 1-phosphate are present in the cytosolic fraction, and that subsequent enzymes, such as digeranylgeranylglyceryl phosphate synthase and some enzymes involved in the conversion of the polar head group are membrane associated (*15*, *16*, *24*). Thus, the biosynthesis of archaeal membrane lipids can be described as follows. *sn*-3-*O*-Geranylgeranylglyceryl 1-phosphate synthesized in the cytoplasm is transferred to the membrane, and a membrane associated digeranylgeranylglyceryl phosphate synthase catalyzes the transfer of a geranylgeranyl group onto 3-*O*-geranylgeranylglyceryl 1-phosphate. Some enzymes involved in the conversion of the polar head group are involved at this step, because CDP-archaeol synthase on the ether phospholipids synthetic pathway is known to be specific for unsaturated archaetidic acid (*16*). Finally, the resulting products, 2,3-digeranylgeranylglycerophospholipids, are hydrogenated by the reductase located in the membrane fraction.

According to our data, this reductase requires FAD and NADH as cofactors, and NADH can be replaced by dithionite. Consequently, the reaction mechanism can be depicted as shown in Fig. 7 and consists of two steps: first the flavin cofactor in the enzyme is reduced by NADH: then the substrate double bonds are hydrogenated by the reduced flavin similar to the mechanism of reductases involved in the biosynthesis of fatty acids and chlorophyll (*25*, *27*).

The N-terminal 20 amino acid sequence is identical to the sequence encoded by the putative geranylgeranyl reductase gene, the Ta0516m gene, in the *T. acidophilum* genome, which clearly indicates that the Ta0516m gene of the *T. acidophilum* genome can be functionally assigned

as digeranylgeranylglycerophospholipid reductase. The Ta0516m gene encodes a protein of 396 amino acids, and molecular mass (43.3 kDa) estimated from the amino acid sequence is well in line with the molecular mass of the reductase (*ca.* 45 kDa from SDS-PAGE). These data confirm that the Ta0516m gene encodes our reductase.

BLAST searches indicated that genes with significant similarity to Ta0516m can be found in many archaeal species whose genomes have been completely sequenced. Since digeranylgeranylglycerophospholipid reductase is essential for the biosynthesis of membrane lipids in archaea, these homologues are considered to be involved in the reduction of digeranylgeranylglyceryl phosphate and its analogues. In addition, it should be noted that homologous genes are also present in some bacteria and eukaryotes (Figs. 8 and 9). These homologues are flavin dependent reductases involved in the biosynthesis of chlorophyll (*25*, *27*). Since the structures of substrates for these geranylgeranyl reductases and digeranylgeranylglycerophospholipid reductase are quite similar, the evolutionary aspects of these reductases might be intriguing.

In summary, we have successfully purified 2,3-di-*O*-geranylgeranylglycerophospholipid reductase from a cell free extract of the thermoacidophilic archaeon *Thermoplasma acidophilum*. The Ta0516m gene of the *T. acidophilum* genome can be functionally assigned as digeranylgeranylglycerophospholipid reductase based on the N-terminal amino acid sequence of the purified enzyme. Furthermore, the present study demonstrates that 2,3-di-*O*-geranylgeranylglycerophospholipid reductase is a membrane associated protein and that the hydrogenation of all double bonds in 2,3-digeranylgeranylglycerophospholipids is catalyzed by a single enzyme.

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