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Purification and Characterization of Geranylgeranylglyceryl Phosphate Synthase from a Thermoacidophilic Archaeon, *Thermoplasma acidophilum*

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We purified a geranylgeranylglyceryl phosphate (GGGP) synthase from *Thermoplasma acidophilum* by several steps of chromatography. Based on the proteinase-fragment-mass-pattern analysis of the SDS-PAGE band of the partially purified protein, the DNA sequence encoding the protein was identified from the whole genome sequence database of the species. The gene encoding GGGP synthase in *T. acidophilum* was cloned after PCR amplification of the gene from the genomic DNA. The recombinant enzyme was expressed in *Escherichia coli* and purified. A single band with a molecular mass of 27 kDa was obtained by SDS-PAGE analysis. The apparent native molecular mass of the enzyme was about 50 kDa based on gel filtration chromatography, suggesting that the enzyme is active as a homodimer. As the GGGP synthase from *Methanobacterium thermoautotrophicum* has been reported as a pentamer, the enzymes of the two organisms have different oligomeric structures. Other characteristics, including substrate specificity, are similar for the GGGPs of these organisms.

Key words: Archaea, ether lipid, geranylgeranylglyceryl phosphate synthase, isoprenoid, *Thermoplasma acidophilum*.

Abbreviations: CHES, 2-cyclohexylaminoethanesulfonic acid; DGGGP, digeranylgeranylglyceryl phosphate; G-1-P, *sn*-glycerol-1-phosphate; G-3-P, *sn*-glycerol-3-phosphate; GGGP, geranylgeranylglyceryl phosphate; GGPP, geranylgeranyl pyrophosphate; GP, glycerophosphate; Mes, morpholinoethanesulfonic acid.

The membrane lipids of Archaea are different from those of Bacteria and Eucarya. One of the most distinguishing features of archaeal lipids is an ether linkage between isopranyl alcohol and glycerol or polyol (*1*). Two fully saturated isopranoid groups of 20 or 25 carbon atoms are attached to a molecule of glycerol, which is called archaeol; two isopranoid groups of 40 carbon atoms attached to two molecules of glycerol at each end, which is called caldarchaeol (*2*). The structures of archaeal membrane lipids were analyzed and determined in methanogens, halophiles and thermophiles (*3*). Isopranoid chains in the ether lipids of Archaea are synthesized through a common pathway in three domains (*4–6*). However, the second half of the biosynthetic pathway of ether lipids is unique in Archaea and much less investigated than isoprenoid synthesis enzymes. Investigation of this unique archaeal membrane-lipid biosynthesis may provide a clue to the early evolution of life. Koga *et al*. have reported that *sn*-glycerol-1-phosphate (G-1-P) dehydrogenase, which determines the enantiomeric specificity of ether lipids, does not show similarity to the eubacterial enzyme, *sn*-glycerol-3-phosphate (G-3-P) dehydrogenase, which is responsible for enantiomeric ester lipid synthesis (*7*). They suggested that G-1-P dehydrogenase and G-3-P dehydrogenase originated from different ancestral enzymes and that this difference is responsible for the divergent evolution of Archaea and Bacteria (*7*).

Ether bond formation proceeds in two steps (Fig. 1). GGGP synthase catalyzes the reaction forming GGGP from G-1-P and geranylgeranyl pyrophosphate (GGPP), and then, digeranylgeranylglyceryl phosphate (DGGGP) synthase catalyzes the reaction forming DGGGP from GGGP and GGPP. The cytosolic fraction contains the GGGP synthase activity, while the membrane fraction contains the DGGGP synthase activity (*8*).

Recently, GGGP synthase from *Methanobacterium thermoautotrophicum*, Marburg strain was purified by a combination of chromatographic steps (*9*), and the gene encoding GGGP synthase was cloned using probes designed from the N-terminal sequence (*10*). This enzyme has a molecular mass of 29 kDa, and is a cytosolic enzyme that is active as a homopentamer. G-1-P is the best acceptor of GGPP to form ether bonds in ether lipids. The substrate specificities of GGGP synthases from a methanogenic and a halophilic archaea are similar, suggesting a common biosynthetic pathway (*11*). The characterization of GGGP synthase from a thermophile has not yet been attempted.

T. acidophilum is a cell wall-less thermoacidophilic archaeon that grows optimally at 56°C, pH 1.8. The full genome sequence of this organism has been reported by Ruepp *et al*. (http://www.biochem.mpg.de/baumeister/genome/) (*12*). The structures of polar lipids in this organism have been analyzed thoroughly (*13*). The bio-

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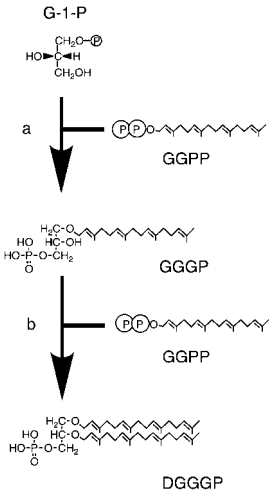

Fig. 1. **Ether bond formation reaction between *sn*-glycerol-1-phosphate and geranylgeranyl pyrophosphate in Archaea.** The reactions are catalyzed by (a) GGGP synthase and (b) DGGGP synthase.

synthetic reaction from an archaeol type polar lipid to a caldarchaeol type polar lipid has been analyzed in this organism (*14*, *15*). Here, we report the purification, coding gene, and the characterization of GGGP synthase from *T. acidophilum*.

MATERIALS AND METHODS

Materials—[1-^{3}H(N)] GGPP triammonium salt was purchased from NEN Life Science Products. *sn*-G-1, 3-P disodium salt hexahydrate and *sn*-G-3-P di(monocyclohexylammonium)salt (approximately 95% purity) were purchased from Sigma. Alkaline phosphatase (from *E. coli* C75) and silica Gel 60 TLC plates were purchased from Takara Shuzo and Merck, respectively.

Microorganism and Culture Conditions—*T. acidophilum* (strain 122-1B2 from Dr. D.G. Searcy) was grown in the medium described previously (*16*). The organism was cultured in a 30-liter fermentor (Marubishi Model MSJ-N2) containing 20 liters of *T. acidophilum* medium at 56°C. The cells were harvested in the early stationary phase by centrifugation at 5,000 ×*g* for 20 min at 4°C. The supernatant was discarded and the pellet was resuspended and washed twice with distilled water adjusted to pH 2.0 with H_2SO_4. About 7 g or more of cells were recovered from 20 liter-culture. The cells were frozen and stored at –80°C until use.

Enzyme Assay and Product Analysis—The reaction buffer contained 100 mM HEPES-Na, pH 7.0, 5 mM $MgCl_2$, 1 mM DTT, 0.23 μM [^{3}H]GGPP (814.0 GBq/mol) and 0.2 mM G-1, 3-P. One microliter of enzyme solution was added to 99 μl of reaction buffer, and the mixture was incubated at 55°C. The reaction was terminated by adding 10 μl of 60% perchloric acid to the mixture (final concentration 1 M). After neutralization with 10 μl of 1 M KOH, 10 μl of 10× alkaline phosphatase buffer (500 mM Tris-HCl, pH 9.0, 10 mM MgCl2) and 0.5 units of alkaline phosphatase were added to the mixture and the mixture was incubated for 1h at 37°C. Labeled products were extracted from the mixture according to the method of Bligh and Dyer (*17*), that is, 300 μl of methanol and 150 μl of chloroform were added and the reaction mixture was agitated vigorously on a voltex mixer. One hundred fifty microliters of chloroform and 150 μl distilled water were added to the suspension, and the suspension was centrifuged. The lower chloroform phase was collected, and dried under a stream of nitrogen gas. The residue was immediately dissolved in a small amount of chloroform–methanol (2:1, v/v) and analyzed by TLC. Hexane-ethyl acetate (1:1, v/v) was used for TLC development (*8*). Radioactive spots of the products on the TLC plate were detected by autoradiography with medical X-ray film (Konica) after fluorometric enhancement with EN^{3}HANCE (NEN Life Science). Each spot was scraped off the plate, the contents were extracted, and the activity was estimated on a liquid scintillation counter LSC-1000 (Aloka) with scintillation cocktail Scintisol AL-1 (Dojindo).

The concentration of substrate GP was varied from 50 to 2000 μM with a fixed concentration of [^{3}H]GGPP (460 nM) to estimate the K_m for GP. The concentration of GGPP was varied from 14.4 to 460 nM, with a fixed concentration of GP (2 mM) to estimate the K_m for GGPP.

Purification of GGGP Synthase from T. acidophilum Cells—Frozen *T. acidophilum* cells were suspended in 30 ml of extraction buffer (25 mM Tris-HCl, pH 8.8, 5 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 0.01% Triton X-100), and disrupted by sonication for 10 min at 0°C. The homogenate was centrifuged at 3,700 ×*g* for 20 min at 4°C, then the supernatant was ultracentrifuged at 60,000 ×*g* for 20 min at 4°C.

The final supernatant was applied to a DEAE-cellulose column (DE 52; Whatman) equilibrated with 200 ml of buffer A (25 mM Tris-HCl, pH 8.8, 0.5 mM EDTA, 0.01% Triton X-100). After the column was washed with 100 ml of buffer A, proteins were eluted with a 50 mM step gradient from 50 to 250 mM NaCl in buffer A. The protein precipitated between the 30 and 80% saturating concentrations of $(NH_4)_2SO_4$ was dissolved in 10 ml of buffer B (25 mM BisTris, pH 7.0, 0.5 mM EDTA, 0.01 % Triton X-100) containing 15% $(NH_4)_2SO_4$ (w/v) and loaded onto a Phenyl-650S column (Toso) equilibrated with buffer B containing 15% $(NH_4)_2SO_4$ (w/v). The column was washed with buffer B containing 15% $(NH_4)_2SO_4$ and eluted with a 15–0% linear $(NH_4)_2SO_4$ gradient. The active fractions were pooled and dialyzed against buffer C (25 mM Tris-HCl, pH 8.3, 0.5 mM EDTA, 0.01% Triton X-100) and loaded onto a Resourse Q column (Amersham-Pharmacia Biotech) equilibrated with buffer C. The column was washed with buffer C, and eluted with 500 mM NaCl in buffer C. The active fraction was loaded onto a Resourse 15-phenyl column (Amersham-Pharmacia Biotech) equilibrated with buffer B containing 15% $(NH_4)_2SO_4$. The column was washed with buffer B containing 15% $(NH_4)_2SO_4$ and eluted with a 15–0% linear $(NH_4)_2SO_4$ gradient. The active fractions were dialyzed against buffer A and loaded onto a Mini Q column (Amersham-Pharmacia Biotech) equilibrated with buffer A. The column was washed with buffer A and eluted with a linear gradient of 0–1 M NaCl in buffer A. The active fractions were loaded onto a Superdex 200 HR 10/30 column

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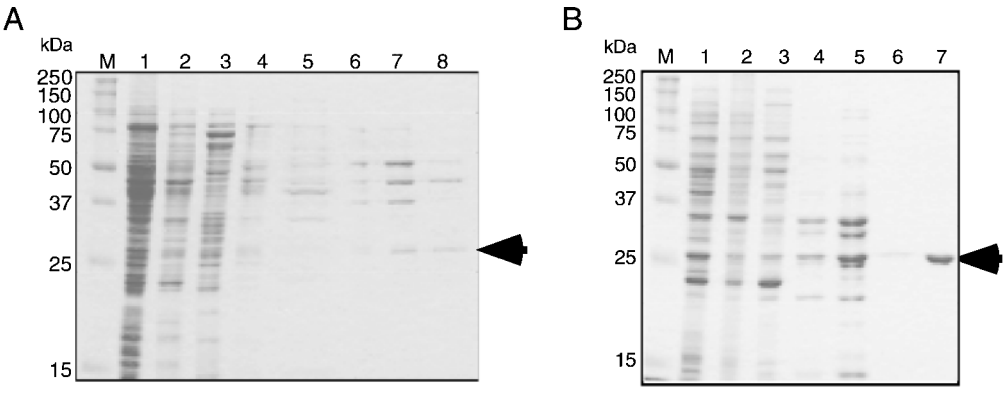

Fig. 2. **SDS-PAGE analysis of active fractions during the purification of *T. acidophilum* GGGP synthase.** Panel A, 12% SDS-PAGE at each step in the purification of GGGP synthase from *T. acidophilum* cells. The gels were stained with Coomassie Brilliant Blue R-250. Lane M, Protein standard marker; 1, crude extract; 2, DE 52; 3, $(NH_4)_2SO_4$ precipitate; 4, Phenyl-650S; 5, Resourse Q; 6, Resourse 15-phenyl; 7, Mini Q; 8, Superdex 200. Panel B, 12% SDS-PAGE at each step in the purification of recombinant GGGP synthase. Lane M, Protein standard marker; 1, crude extract of *E. coli* BL21-Codon Plus (RIL)/pTA995; 2, supernatant from a crude extract of *E. coli* cells before heat treatment; 3, supernatant from a crude extract after heat treatment; 4, DE 52; 5, $(NH_4)_2SO_4$ precipitate; 6, Phenyl-650S; 7, Resourse Q. Arrows indicate the target bands.

(Amersham-Pharmacia Biotech) equilibrated and eluted with buffer A containing 100 mM NaCl.

Protein concentration was determined with a BCA protein assay reagent (Pierce) using bovine serum albumin as a standard. Protein fractions were analyzed by SDS-PAGE. Protein bands were stained with Coomassie Brilliant Blue R-250.

Mass Spectral Analysis—About 30 pmol of purified GGGP synthase from SDS-PAGE was subjected to reductive alkylation with iodoacetoamide, digested with trypsin and used for peptide mass analysis with a MALDI-TOF MASS (Shimadzu Biotech).

Cloning of GGGP Synthase Gene from T. acidophilum—The primers were designed on the basis of the genomic sequence of *T. acidophilum*. Two synthetic oligonucleotides, 5′-ATGATGACCGTCCTCGAAGAC-3′ and 5′-TCATTGTATTTTTGATATTCCTACC-3′, were used as primers for amplification of the GGGP synthase gene. PCR was carried out with *T. acidophilum* genomic DNA as a template. The initial denaturation step was 5 min at 95°C, followed by 25 cycles of 1 min at 94°C, 30 sec at 55°C, and 1 min at 72°C. The PCR product was cloned into a pCR T7/CT-TOPO vector (Invitrogen).

Expression and Purification of Recombinant GGGP Synthase—E. coli BL21-Codon Plus (DE3)-RIL (Stratagene) were transformed with the plasmid pTA995 and grown in Luria-Bertani medium containing 100 μg/ml ampicillin at 37°C. When the cell density reached OD_{600} = 0.8, IPTG (isopropyl β-D-thiogalactoside) was added to a final concentration of 1 mM and the culture was continued for an additional 3 h before the cells were harvested by centrifugation. The recombinant enzyme was purified by a procedure similar to that described above using the same buffer solution. Ten grams of cells were suspended in 30 ml of extraction buffer. The cells were disrupted by sonication, and cell debris was removed by centrifugation. The cell-free extract was incubated at 55°C for 20 min, and cleared by centrifugation. The supernatant was applied to a DEAE-cellulose column as described above. The sample precipitating between 30 and 80% saturating $(NH_4)_2SO_4$ was dissolved in 10 ml of buffer B and purified on Phenyl-650S and Resourse Q columns. The active fractions were concentrated with a Microcon YM-30 (Millipore) microfilter to approximately 1 mg/ml. The enzyme was stored at 4°C until use.

Native Molecular Mass Determination—A 10 μl sample was mixed with 90 μl of buffer D (25 mM BisTris, pH 7.0, 5 mM $MgCl_2$, 5 mM ME (2-mercaptoethanol), 150 mM NaCl, 0.01% Triton X-100), applied to a Superdex 200 HR 10/30 column, and eluted with buffer D at a flow rate of 0.2 ml/min.

Sequence Alignments and Phylogenetic Analysis of Putative GGGP Synthases—Putative amino acid sequences for GGGP synthase were searched in DDBJ (DNA database of Japan; http://www.ddbj.nig.ac.jp/) and aligned with the ClustalX program. Phylogenetic trees were inferred by the neighbor-joining distance matrix method using the interface provided by DDBJ. Bootstrap values were estimated by calculating for 1000 repetitions of resampling.

Table 1. **Purification of GGGP synthase from *T. acidophilum* cells.**

Steps	Protein (mg)	Activity (nmol·min^{-1})	Recovery (%)	Specific activity (nmol·min^{-1}·mg^{-1})	Purification (fold)
Extracts	489	209	100	0.4	1
DE 52	221	328	64	1.5	3.8
15–80% AS	97	58	28	0.6	1.5
Phenyl 650S	24	187	90	7.8	20
Resourse Q	1.6	442	210	280	700
Res Phenyl	1.4	150	72	110	275
Mini Q	0.3	34	16	110	275
Superdex 200	0.08	30	14	380	950

Results from 7.1 g (wet weight) of cells.

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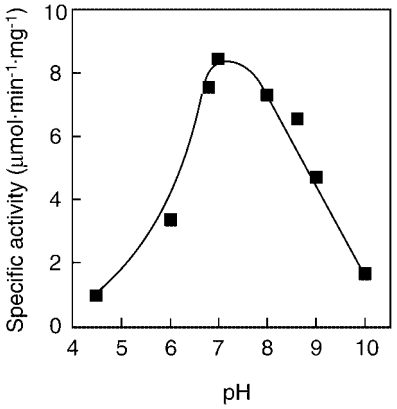

Fig. 3. **pH dependence of GGGP synthase.** Buffers used were sodium acetate (pH 4.5), Mes (pH 6.0), HEPES (pH 6.8, 7.0, 7.4, and 8.0), Tris (pH 8.6), CHES (pH 9.0 and 10.0) at the same concentration as the standard reaction buffer. Reactions were carried out at 45°C.

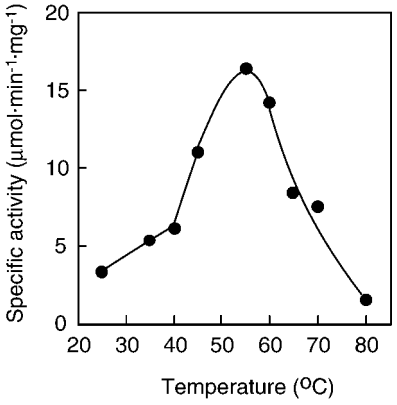

Fig. 4. **Temperature dependence of GGGP synthase.**

RESULTS AND DISCUSSION

Purification of GGGP Synthase from T. acidophilum Cells—GGGP synthase was purified from *T. acidophilum*. The purification process is summarized in Table1. Although the enzyme was purified over 900-fold, three bands were still seen on SDS-PAGE (Fig. 2A). Based on the similarity of the molecular mass to that of GGGP synthase from *M. thermoautotrophicum* (29 kDa), the band migrating at molecular mass 27 kDa was estimated to be GGGP synthase. The 27 kDa band was reproducibly detected in samples of independent purifications, supporting the assignment.

Peptide Mass Finger Printing Analysis of GGGP Synthase—GGGP synthase was subjected to reductive alkylation by iodoacetoamide, digested with trypsin and subjected to peptide mass finger printing analysis. The fragment mass pattern was compared with estimated patterns of ORFs (open reading frames) in genetic databases. The best score was obtained for ORF NP_394455 (TA0995 in the *T. acidophilum* full genome sequence database), which encodes a hypothetical protein consisting of 253-amino acid. Although the reaction of the next enzyme in the biosynthetic pathway, DGGGP synthase, is expected to be related to that of GGGP synthase, BLAST search using GGGP synthase gene as a key sequence against the *T. acidophilum* genome revealed no similarity to any ORF.

Expression of Recombinant GGGP Synthase—Primers were designed for ORF TA0995. PCR was done using these primers and the *T. acidophilum* genome DNA as the template. A DNA fragment with the expected length (about 750 bp) was amplified, and cloned in a pCR T7/CT-TOPO vector. Sequence analysis of the cloned fragment confirmed the full ORF TA0995 in the vector, and the plasmid was named pTA995.

The recombinant GGGP synthase was expressed in *E. coli* BL21 (DE3) Codon Plus (RIL) cells harboring the pTA995 plasmid. The *E. coli* cells harboring the plasmid were grown until mid-log phase and gene expression was induced by IPTG. SDS-PAGE analysis of a cell-free extract of the induced cells revealed an additional 27 kDa band, corresponding to the calculated molecular mass of the putative GGGP synthase. Recombinant GGGP synthase was purified to homogeneity by a five-step purification procedure (Fig. 2B).

Optimum Catalytic Conditions for Recombinant GGGP Synthase—Purified GGGP synthase was active over a wide range of pH with an optimum at around pH 7 (Fig. 3). Although the optimum pH for the growth of *T. acidophilum* is pH 1–2, the internal pH is close to neutral (pH 6.2) (*18*). The optimum pH of the enzyme is about 1 pH unit above the internal pH of *T. acidophilum*. The temperature dependence of the enzymatic reaction is shown in Fig. 4. Maximum catalytic activity was found at around 55°C, which corresponds to the optimal growth temperature of *T. acidophilum*.

The optimum concentration of Mg^{2+} was 5–10 mM and the enzymatic activity was slightly inhibited by higher concentrations of Mg^{2+} (Fig. 5). GGGP synthase was inactive in the presence of EDTA (Table 2). Mn^{2+} and Zn^{2+} could not replace Mg^{2+} at any concentrations tested (Fig. 5). GGGP synthase from *M. thermoautotrophicum* also requires about 4 mM Mg^{2+} (*9*, *19*). Isoprenoid elongation enzymes require Mg^{2+} for the catalytic activity (*4*). The crystal structure of the *cis*-prenyl chain elongating enzyme, undecaprenyl diphosphate synthase, indicates that a magnesium bridge can form between the carboxyl group of an aspartic acid residue and the diphosphate part of the allylic substrate, farnesyl pyrophosphate (*20*).

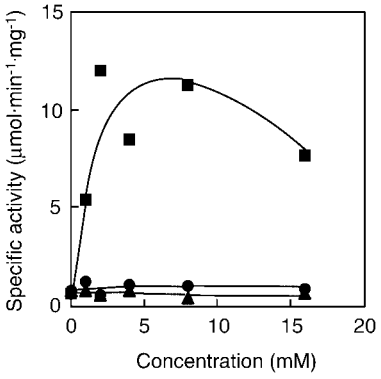

Fig. 5. **Divalent cation dependence of GGGP synthase.** Solid squares, Mg^{2+}; solid circles, Mn^{2+}; solid triangles, Zn^{2+}. Reactions were carried out at 45°C

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Table 2. **Effects of ingredients in the reaction mixture.**

Added or removed from the reaction mixture	Relative activity (%)
Triton X-100 0.1%	100
Triton X-100 0.01%	112.1 ± 17.6
Triton X-100 0.001	36.7 ± 6.3
Triton X-100 0%	5.9 ± 2.9
–DTT	80.2 ± 6.2
10 mM EDTA	≪0.01
–G-1, 3-P, +G-3-P	5.6 ± 1.5

Basic reaction mixture contained 100 mM HEPES, pH 7.0, 5 mM $MgCl_2$, 1 mM DTT, 0.23 μM [^{3}H]GGPP, 0.2 mM G-1, 3-P, and 0.1 % Triton X-100 unless otherwise stated. Data are presented as M ± SE of triplicate experiments.

Because both isoprenoid elongation enzyme and GGGP synthase use an isoprenoid pyrophosphate as a substrate, Mg^{2+} may play similar roles in both enzymes.

Essential components for the reaction were investigated (Table 2). Triton X-100 was essential for optimum activity. The isoprenoid elongation enzyme undecaprenyl pyrophosphate synthase also requires Triton X-100 (*21*). The release of the hydrophobic product from the enzyme is inhibited in the absence of Triton X-100 in the enzyme (*21*). After the reaction carried out by GGGP synthase, the following second ether-bond-formation step is catalyzed by a membrane protein, DGGGP synthase (*8*). Triton X-100 may be needed to release the product, GGGP, to the cell membrane for the next ether-bond-formation step. Triton X-100 may substitute for membrane lipids as found in the case of undecaprenyl pyrophosphate synthase (*22*).

When the substrate G-1, 3-P was replaced with G-3-P, the enzymatic activity was only 5.6% of that for G-1, 3-P. Thus GGGP synthase is specific for G-1-P, and the residual activity can be attributed to the impurity G-3-P (95% purity). Specificity for G-1-P as a substrate has also been reported for GGGP synthases from *M. thermoautotrophicum* (purified enzyme) (*9*) and *Halobacterium halobium* (crude extract) (*11*).

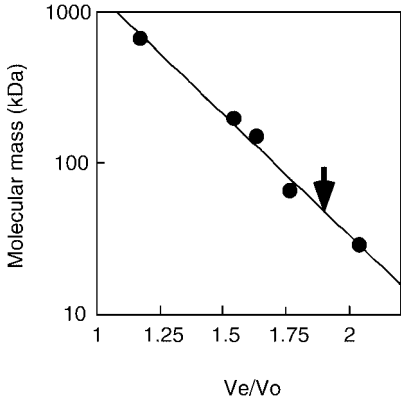

Fig. 6. **Determination of the molecular mass of GGGP synthase by gel filtration.** Protein concentration was monitored at 280 nm. Marker proteins were thyrogloblin (669 kDa), β-amylase (200 kDa), alcohol dehydrogenase (150 kDa), bovine serum albumin (66 kDa), carbonic anhydrase (29 kDa). The relative retention volume of GGGP synthase is indicated by an arrow.

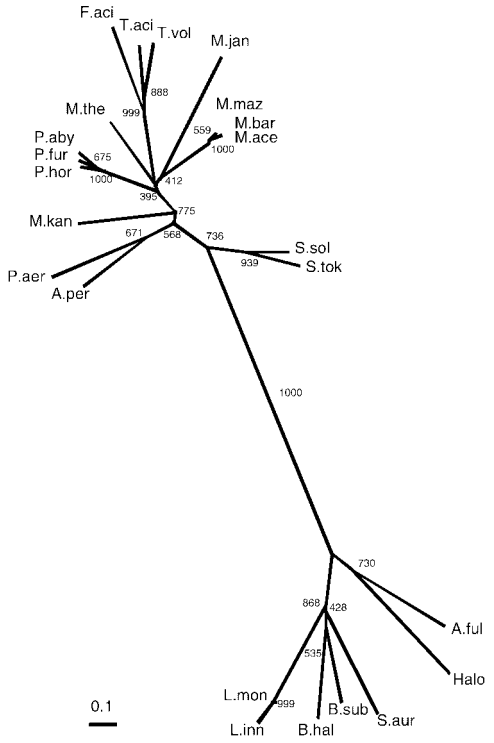

Fig. 7. **Phylogenetic tree of putative GGGP synthase sequences identified from a BLAST search.** The scale bar represents 0.1 substitutions per amino acid position. Abbreviations and accession numbers are as follows: T.aci, *T. acidophilum* (NP_394455); T.vol, *Thermoplasma volcanium* (NP_111253); F.aci, *Ferroplasma acidarmanus* (ZP_00000895); P.hor, *Pyrococcus horikoshii* (NP_143028); P.aby, *Pyrococcus abyssi* (NP_126789); P.fur, *Pyrococcus furiosus* (NP_578768); M.bar, *Methanosarcina barkeri* (ZP_00077569); M.the, *M. thermautotrophicus* (NP_275695); M.maz, *Methanosarcina mazei* (NP_632967); M.ace, *Methanosarcina acetivorans* (NP_618839); M.kan, *Methanopyrus kandleri* (NP_613927); M.jan, *Methanocaldococcus jannaschii* (NP_248246) S.sol, *Sulfolobus solfataricus* (NP_341809); S.tok, *Sulfolobus tokodaii* (NP_376171); A.per, *Aeropyrum pernix* (NP_147371); P.aer, *Pyrobaculum aerophilum* (NP_560452); A.ful, *Archaeoglobus fulgidus* (AAB90827); Halo, *Halobacterium* sp. NRC-1 (AAG18849); B.sub, *B. subtilis* (CAB12480); B.hal, *Bacillus halodurans* (BAB04366); S.aur, *S. aureus* (BAB58068); L.mon, *L. monocytogenes* (CAC99838); L.inn, *Listeria innocua* (CAC97102).

Catalytic Properties—The enzyme activity of the purified recombinant GGGP synthase was measured under optimal reaction conditions in 100 mM HEPES, pH 7.0, 1 mM DTT, 5 mM $MgCl_2$, 0.01% Triton X-100 and 2 mM G-1, 3-P, or 0.23 μM GGPP at 55°C. V_{max} was 13.5 ± 2.2 μmol·min^{-1}·mg^{-1} (k_{cat} = 6.1 ± 1.1 s^{-1}) and K_m values were 21.2 ± 1.2 μM for G-1, 3-P, and 75 ± 6 nM for GGPP. The values of the recombinant enzyme from *M. thermoautotrophicum* are V_{max} 4.0 ± 0.1 μmol·min^{-1}·mg^{-1} (k_{cat} = 0.34 ± 0.03 s^{-1}), K_m for G-1-P 13.5 ± 1.0 μM, and K_m for GGPP 506 ± 47 nM at 55°C (*10*). All kinetic values of the GGGP synthase from *T. acidophilum* are comparable to those of the enzymes from *M. thermoautotrophicum*.

Molecular Mass—Gel filtration of the purified recombinant GGGP synthase revealed a molecular mass of 50 kDa (Fig. 6). The molecular mass of the subunit was 27 kDa by SDS-PAGE (Fig. 2B) and is consistent with that estimated from the DNA sequence. Accordingly, GGGP

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Fig. 8. **Alignment of the amino acid sequence of *T. acidophilum* GGGP synthase and homologues from five species of Archaea and two species of Bacteria.** Abbreviations are the same as in the legend to Fig. 7. Strictly conserved residues and similar residues are indicated by asterisks and dots, respectively.

synthase from *T. acidophilum* is expected to be a dimer. The native molecular mass of GGGP synthase from *M. thermoautotrophicum* is 137 kDa as determined by equilibrium centrifugation, and the enzyme is expected to be a pentamer (*10*). Thus, the GGGP synthases from *T. acidophilum* and *M. thermoautotrophicum* are different in the subunit structure. Although the active form of GGGP synthase is only known in two species, an investigation of GGGP synthases from several other Archaea may reveal the evolutionary history of the active form of GGGP synthase.

Analysis of Amino Acid Sequence for GGGP Synthase—BLAST search was performed to identify sequences showing similarity to GGGP synthase. Genes with significant similarity were identified in eighteen archaeal species, and these were divided into two groups by phylogenetic analysis (Fig. 7), one including sixteen archaeal sequences, and the other two archaeal sequences (*Archaeoglobus fulgidus* and *Halobacterium* sp. NRC-1). The sequences in the first group have mutual sequence identity of 35% or more. The sequences in the second group also have mutual sequence identity of 35% or more. Between the two groups, however, the sequences show lower identity (30% or less). Although the GGGP synthase activity of the products of the second group was not tested, GGGP synthase activity was observed *in vitro* in *H. halobium* (*11*). Accordingly, the sequences in the second group are likely to encode GGGP synthase. These two archaeal GGGP synthases show high homology to the *pcr*B gene sequence in Bacteria. The *pcr*B gene product has no known function (*23*) and has only been found in a few Bacterial species, including *Bacillus subtilis, Staphylococcus aureus, Listeria monocytogenes.* It is interesting to examine the functional relationship between PcrB and GGGP synthase.

The presence of a conserved (V)GGG(I)(R) motif near residue 230 (Fig. 8) was pointed out by Soderberg *et al.* (*10*). We found that the motif is also conserved in the Bacterial sequences (Fig. 8). A similar motif is found in the phosphate-recognition loop of a histidine biosynthetic protein, HisF (*24*). This motif is suggested to be responsible for the recognition of a phosphate in *N*′-[(5′-ribulosyl)-formimino]-5-aminoimidazole-4-carboxamide-ribonucleotide to produce imidazole glycerol phosphate and 5-aminoimidazole-4-carboxamide-1-β-D-ribofuranosyl 5′-monophosphate with HisH. The motif may be also responsible for the recognition of a phosphate group on GGPP or G-1-P by members of the first group. The motif is also conserved in the sequence of members of the second group, accordingly, these gene products may be involved in the reaction of a substrate with a phosphate group.

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