The first pathway-specific step in the biosynthesis of the core membrane diether lipids in archaeabacteria is the alkylation of the primary hydroxyl group in (S)-glyceryl phosphate by geranylgeranyl diphosphate. The reaction is catalyzed by (S)-3-O-geranylgeranyl glyceryl phosphate ((S)-GGGP) synthase. The cytosolic enzyme was purified to homogeneity from the moderately thermophilic archaeabacterium *Methanobacterium thermoautotrophicum* by a combination of ammonium sulfate precipitation, four chromatographic steps (DE52, Q-Sepharose, phenyl-Sepharose, and Protein Pak), and native polyacrylamide gel electrophoresis. SDS-polyacrylamide gel electrophoresis of gel-purified GGGP synthase gave a single band at 29 kDa. The enzyme requires Mg²⁺ for optimal activity, although prenyltransferase is also seen in buffers containing Mn²⁺ or Zn²⁺. A well-defined pH optimum occurs between 6.0 and 7.5. Maximal activity is seen at 50-65 °C. The Michaelis constants for GGGP synthase are *V*_max = 4.1 ± 0.5 μmol min⁻¹ mg⁻¹, *K*_M(GGGP) = 4.1 ± 1.1 μM, and *K*_M(GP) = 41 ± 5 μM.

Archaeabacteria are sufficiently different from other forms of life to be classified as a distinct kingdom (1). These unusual organisms normally inhabit hostile environments characterized by high concentrations of salt, high temperatures, low pH, or the complete lack of oxygen and can resemble eubacteria, eukaryotes, or neither, depending on which phenotypic characteristics are considered (2). Archaeabacteria have distinctive biochemical features as well, including highly diverged ribosomal RNAs, unique metabolic cofactors, and novel membrane lipids (2).

Archaeabacterial membranes contain lipids consisting of saturated isoprenoid chains attached to glycerol by ether linkages (3). The core membrane diethers contain two phytane moieties and form bilayers in much the same way as fatty acid esters form bilayers in other organisms. In addition, thermophilic archaeabacteria contain unique cyclic tetraethers that are derivatives of the primary hydroxyl in (S)-glyceryl phosphate (GP) by GGGP. The other is microsomal and catalyzes alkylation of the remaining hydroxyl moiety in the monoether by a second GGGP, as shown in Scheme 1. A similar pathway was found in *H. halobium* (11). The alkylation of GP by GGGP is a prenyltransferase reaction where a hydroxyl group is the nucleophile in the acceptor substrate. Except for the biosynthesis of archaeabacterial lipids, there are only a few examples of prenyltransferases reacting with oxygen-containing acceptors (14, 15), and none of the enzymes responsible for these reactions has been characterized. We now report the purification and catalytic properties of (S)-3-O-geranylgeranyl glyceryl phosphate (GGGP) synthase, the enzyme that catalyzes the first pathway-specific step in the biosynthesis of archaeabacterial core membrane lipids.

**EXPERIMENTAL PROCEDURES**

**Materials**—Frozen cells of *M. thermoautotrophicum* Marburg were provided by Dr. Lacy Daniels. GGGP, (S)-GP, and (S)-[1-³H]GP were available from an earlier study (15). BHDA was from Pfaltz & Bauer. SDS was from Bio-Rad. Ultrapure acrylamide and (NH₄)₂SO₄ were from ICN Biochemicals. Other materials were from Sigma unless specified otherwise.

**General Procedures**—Dialyses were for 4 h in *M*, 6,000-8,000 cut-off tubing or 2 h in *M*, 12,000-14,000 cut-off tubing (Spectrum). Centrifugations were performed at 20,000 × *g* (JA-20 rotor) or 10,000 × *g* (JA-14 rotor) in a Beckman TJ-6 centrifuge. A Pharmacia fast protein liquid chromatograph was used for chromatography. Protein samples were

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1 The abbreviations used are: GGGP, geranylgeranyl diphosphate; BHDA, bicyclo[2.2.1]hept-5-ene-2,3-dicarboxylic anhydride; BisTris, bis[2-hydroxyethyl]iminotriis(hydroxymethyl)methane; 2-ME, 2-mercaptoethanol; CAPS, 3-(cyclohexylamino)propanesulfonic acid; GGGP, 3-geranylgeranyl glyceryl phosphate; GP, 1-glyceryl phosphate; PAGE, polyacrylamide gel electrophoresis; PIPES, piperazine-N,N′-bis(2-ethanesulfonic acid).
(S)-Geranylgeranylgylycerol Phosphate Synthase

The Journal of Biological Chemistry

HR 10/10 column (Pharmacia) equilibrated with 100 ml of starting buffer (50 mM phosphate, 5 mM 2-ME, and 1.0 M ammonium sulfate, pH 7.0). The column was washed with starting buffer and eluted with a 180-ml linear gradient of 0–1 M (NH₄)₂SO₄ at 1 ml/min. The active fractions were pooled, dialyzed against 2 x 2 liters of 25 mM BisTris acetate and 5 mM 2-ME, pH 7.0, and loaded onto a 0.75 x 7.5 cm Protein Pak column (Waters) equilibrated with 1 ml/min of starting buffer (25 mM BisTris acetate and 5 mM 2-ME, pH 7.0). The column was eluted with 100 ml of a linear gradient of 0–1 sodium acetate in starting buffer at a flow rate of 0.5 ml/min. The active fractions were pooled.

Native PAGE was performed on a 10% gel as described for stacking SDS-PAGE except that SDS and 2-ME were omitted in all buffers, and samples were not boiled. The gel was pre-run for 20 min. Samples were dialyzed against 10 mM NH₄HCO₃ and concentrated on a SpeedVac. After electrophoresis at a constant current (30 mA) for 40 min, the gel was rinsed with water and soaked in 0.3 M CuCl₂ for 5 min (18). The gel was rinsed with water again, and protein bands were visualized on a black background. Those regions containing protein were sliced from the gel, soaked in 25 mM Tris-Cl and 10 mM EDTA, pH 9.0, for 10 min, and then cut into small pieces. Proteins in individual fractions were electroeluted into 40 mM Tris acetate, pH 8.2, using a Schleicher & Schuell Elutrap and assayed for GGGP synthase activity.

Native Molecular Mass—A Superdex 75 HR 10/10 column (Pharmacia) was eluted at 0.2 ml/min with 50 mM phosphate, 5 mM 2-ME, and 150 mM NaCl, pH 7.0. Standards were aldehyde dehydrogenase (150 kDa), bovine serum albumin (68 kDa), pepsin (34.7 kDa), and cytochrome c (12.4 kDa). A 0.9 x 80 cm Sephacryl S-200 HR column (Pharmacia) was eluted at 0.1 ml/min with 100 mM phosphate and 10 mM 2-ME, pH 7.0. Mass standards were bovine serum albumin (66 kDa), phosphorylase (97.4 kDa), pepsin (34.7 kDa), and cytochrome c (12.4 kDa). Fractions were collected and assayed for GGGP synthase activity.

Metal Ion, pH, and Temperature Dependence of GGGP Synthase—All assays contained 20 µM GGGP, 100 µM GP, and 17.5 ng of GGGP synthase that had been purified through the Protein Pak step. The samples were incubated for 5 min. For studies of the effect of metal ions, the enzyme was first dialyzed against 50 mM phosphate, 10 mM 2-ME, and 5 mM EDTA, pH 7.0, and then against the same buffer without EDTA. The pH studies were in 50 mM buffer containing 10 mM 2-ME and 3 mM MgCl₂. The pH was measured at the reaction temperature. For temperature dependence studies, the enzyme was assayed in 50 mM BHDA, 10 mM 2-ME, 3 mM MgCl₂, pH 7.0.

Kinetic Constants—Initial velocities were measured for varied concentration of one substrate in the presence of a saturating concentration of the other in 50 mM BHDA, 10 mM 2-ME, 3 mM MgCl₂, and 0.1 mg/ml bovine serum albumin, pH 7.0. Assay mixtures were pre-equilibrated at 60°C; and the reaction was initiated by addition of GGGP synthase. Km and Vₘₜₐₓ were calculated using Enzfitter™.

RESULTS

Purification of GGGP Synthase—The purification of GGGP synthase is summarized in Table I. The 50–80% (NH₄)₂SO₄ fraction from cell-free homogenates normally gave a 1.5-fold purification with 70% recovery of activity. Chromatography on DE52 (Fig. 1) gave a broad peak, and attempts to improve resolution were unsuccessful. However, combination and dialysis of the active fractions gave an excellent recovery of units with an almost 10-fold purification. The sample was chromatographed on Q-Sepharose, as shown in Fig. 2, using a linear gradient of (NH₄)₂SO₄ in starting buffer at a flow rate of 0.5 ml/min. The active fractions were pooled.

<table>
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* Assays were done in 50 mM BHDA buffer, pH 7.0, containing 10 mM 2-ME, 3 mM MgCl₂, 20 µM GGGP, and 100 µM (S)-GP at 60°C.
Radioactivity incorporated into Cl, 5 mM 2-ME, and 2% ethylene glycol, pH 7. Buffer B consisted of 1 M NaCl followed by a 500-ml linear gradient of 0.25-0.5 M NaCl in buffer A. Elution was accomplished with a 200-ml linear gradient of 0.25 M NaCl in buffer A, pH 7.0. Elution was accomplished with a 350-ml 280 nm chromatography on Protein Pak (Fig. 4), activity for GGGP linear gradient of sodium acetate. 

As illustrated in Fig. 6, the specific activity of GGGP synthase increased with increasing Mg2+ concentrations up to a saturating value of 4 mM. At low concentrations, Mn2+ was more effective than Mg2+, but higher concentrations of Mn2+ were inhibitory. 

Maximal activity of GGGP synthase was seen between 50 and 65 °C (see Fig. 7) at temperatures in the normal range for optimal growth of M. thermoautotrophicum. At higher temperatures, the enzyme is irreversibly denatured. The pH-rate profile shown in Fig. 8 is a bell-shaped curve with a maximum between 6.0 and 7.5. Kinetic constants were determined at 60 °C in 50 mM BHDA, 10 mM 2-ME, and 3 mM MgCl₂, pH 7.0. Under these conditions, K_Mg = 4.3 ± 1.1 μM, K_Mn = 41 ± 5 μM, and V_max = 4.1 ± 0.5 μmol min⁻¹ mg⁻¹.

**DISCUSSION**

Prenyltransferases catalyze the electrophilic alkylation of electron-rich acceptors by allylic isoprenoid diphosphates with concomitant expulsion of a proton and PP_i. These reactions constitute the major building steps in the isoprenoid biosynthetic pathway. Although prenyl transfer to heteroatoms such as nitrogen, oxygen, or sulfur in acceptor substrates are not uncommon, most work has focused on the enzymes that catalyze alkylation of unsaturated hydrocarbon moieties. Representative reactions of this type are the basic chain elongation catalyzed by farnesyl diphosphate synthase (19), and the synthesis of squalene catalyzed by squalene synthase (21), and alkylation of the aromatic ring in tryptophan by dimethylallyl-tryptophan synthase (22). GGGP synthase catalyzes the first committed step in the biosynthesis of archaeabacterial diether core membrane lipids.
As illustrated in Scheme 1, the substrates for the prenyltransferase are (S)-GP and GGPP (13). Thus, the glyceryl unit in archaeabacterial membrane lipids has the opposite absolute stereochemistry of the glyceryl moiety in the ester lipids from eubacteria and eukaryotes. Biosynthetic studies indicate that GGPP is synthesized from acetyl coenzyme A by the normal mevalonate pathway (9). However, labeling experiments uncovered two different pathways for biosynthesis of the glyceryl moiety. In \textit{Sulfolobus acidocaldarius}, glycerol was incorporated intact (23), whereas glycerol labeled with deuterium at C-2 lost the label when the molecule was incorporated into the membranes of \textit{H. halobium} (24). Zhang and Poulter (11) studied the substrate selectivity for GGPP synthase in cell-free extracts from \textit{H. halobium} and found that (S)-GP is the preferred prenyl acceptor. Thus, the differences detected by the labeling experiments must occur before the prenyl transfer step catalyzed by GGPP synthase.

Cytosolic GGPP synthase from \textit{M. thermoautotrophicum} was purified to >85% homogeneity by a combination of ion exchange and hydrophobic chromatographies. A highly purified sample was obtained by native PAGE. Active GGPP synthase was identified by visualizing the gel with CuCl$_2$, according to the procedure described by Lee \textit{et al.} (18), without destroying enzymatic activity. This procedure allowed us to detect protein bands, and gel slices containing individual protein bands were assayed to determine which contained GGPP synthase. Comparisons of molecular masses determined by SDS-PAGE and gel filtration suggest that GGPP synthase is a homopolymer with four or more subunits. Although a variety of structural motifs, including monomers (25), homodimers (21, 26), and heterodimers (27), have been reported for prenyltransferases, GGPP synthase may be the first example of a higher order aggregate.

Like other prenyltransferases, GGPP synthase required a divalent metal ion for activity (18). Although Mg$^{2+}$ was preferred, prenyltransferase activity was also seen with Mn$^{2+}$ and Zn$^{2+}$. The metal ion dependence for GGPP synthase was similar to that reported for farnesyl diphosphate synthase (28). In the latter case, Laskovics \textit{et al.} (29) concluded that Mg$^{2+}$ was not bound directly to the enzyme but was sequestered along with the substrates as a magnesium salt of the diphosphate moieties.

The mechanism for alkylation of (S)-GP by GGPP appears to be related to the electrophilic reactions seen for farnesyl diphosphate synthase (18) and dimethylallyl tryptophan synthase (21). These enzymes catalyze rupture of the carbon-oxygen bond in the diphosphate-isoprene linkage to generate highly electrophilic allylic carbocations that subsequently alkylate the prenyl acceptors. Formation of the electrophilic intermediates depends on having an allylic double bond in the

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**Fig. 5. PAGE analysis of GGPP synthase.** Panel A, 12% SDS-PAGE of proteins from each step in the purification. Panel B, native PAGE of GGPP synthase. CE, crude extract; AP, (NH$_4$)$_2$SO$_4$ precipitate; DE, DE52; QS, Q-Sepharose; PS, phenyl-Sepharose; PP, Protein Pak; NP, native PAGE-purified; S, protein standards; GGPP, GGPP synthase. Protein standards were as follows: myosin (H chain), 200 kDa; phosphorylase b, 97.4 kDa; bovine serum albumin, 68 kDa; ovalbumin, 43 kDa; carbonic anhydrase, 29 kDa; 3-6-5 lactoglobin, 18.4 kDa; lysozyme, 14.3 kDa.

**Fig. 6. Metal ion dependence of GGPP synthase.** Measured in 50 mM BHDA buffer, pH 7.0, containing 10 mM 2-ME, 20 mM GGPP, and 100 mM (S)-GP. GGPP synthase was dialyzed against buffer containing EDTA before use in the assay.

**Fig. 7. Temperature dependence of specific activity for GGPP synthase.** Measured in 50 mM BHDA buffer, pH 7.0, containing 10 mM 2-ME, 3 mM MgCl$_2$, 20 mM GGPP, and 160 mM (S)-GP.

**Fig. 8. pH-specific activity profile for GGPP synthase.** Buffers used were sodium acetate (□), PIPES (■), triethanolamine (○), and CAPS (○).
prenyl donor to provide the reactivity required to generate the carbocation. Zhang and Poulter (13) found that cytosolic preparations from *M. thermoautotrophicum* catalyze alkylation of (S)-GP by phytanyl diphosphate, a derivative of GGPP with a single double bond at C-2, at a slower but significant rate. However, the fully saturated derivative, phytanyl diphosphate, is inactive. Thus, the mechanism for prenyl transfer of a geranylgeranyl group from GGPP to the hydroxyl group in GP appears to be an electrophilic substitution similar to those that have been established for other prenyltransferases.

**Acknowledgement**—We thank Professor Lacy Daniels for providing freshly frozen samples of *M. thermoautotrophicum*.

**REFERENCES**