Biosynthesis of Archaebacterial Lipids in *Halobacterium halobium* and *Methanobacterium thermoautotrophicum*

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The core membrane lipids in archaebacteria are isoprenoid ether derivatives of glycerol instead of fatty acid esters found in other organisms. Activities for three key enzymes in membrane lipid biosynthesis, isopentenyl diphosphate (IPP) isomerase, geranylgeranyl diphosphate (GGPP) synthase, and 3-*O*-geranylgeranylglyceryl phosphate (GGGP) synthase were found in the cytosolic fractions of cell-free homogenates from the strict anaerobe *Methanobacterium thermoautotrophicum* and the extreme halophile *Halobacterium halobium*. The substrate selectivities of GGGP synthase from both sources were similar and indicate a common pathway for biosynthesis of the isoprenoid compounds in core membrane lipids from methanogenic and halophilic archaebacteria.

Archaebacteria are unique organisms that diverged from other life forms at the earliest stages of evolution.[1,2] They inhabit normally hostile environments characterized by high salt, low pH, high temperatures, or complete lack of oxygen.[2] Archaebacteria also have distinctive biochemical features that include highly diverged 5S and 16S RNAs,[3-5] unique metabolic cofactors,[6-9] and novel core membrane lipids.[10-13] Archaebacterial membranes consist of glyceryl ethers bearing saturated isoprenoid side chains.[10-15] Diethers in thermophilic species are joined covalently at the ends of the isoprene chains to form dimeric membrane spanning tetraethers.[11,14]

In *Methanobacterium thermoautotrophicum* the membrane diethers are constructed from (*S*)-glyceryl phosphate (GP)[16] and geranylgeranyl diphosphate (GGPP) by two prenyl transferases.[17] The first, 3-*O*-geranylgeranylglyceryl phosphate (GGGP) synthase, converts (*S*)-GP to (*S*)-GGGP, and the second catalyzes alkylation of the secondary hydroxyl in (*S*)-GGGP by another molecule of GGPP. GGGP synthase strongly prefers (*S*)-GP as a prenyl acceptor over (*R*)-GP or dihydroxyacetone phosphate.

The absolute stereochemistry of the glyceryl moeity in all archaebacterial diethers is opposite that of glyceryl ester membrane lipids in eubacteria and eukaryotes.[18,19] Incorporation of labeled glycerol or glycerol derived from glucose into core membrane diethers by the thermoacidophile *Sulfolbus acidocaldarius* indicated that the *pro-R* hydroxymethyl group is prenylated and that the C(2) hydrogen is retained.[20,21] These observations are consistent with a pathway where glycerol is stereospecifically phosphorylated to (*S*)-GP, which is then the substrate for GGGP synthase in analogy with studies at the cell-free level in *M. thermoautotrophicum*.[17] However, another pathway operates in the extreme halophile *Halobacterium halobium*. In this organism the *pro-S* hydroxymethyl of glycerol is alkylated, and the hydrogen at C(2) is lost upon incorporation of the C_3 unit into the membrane diethers.[22-24] Kakinuma et al.[23] suggested that inversion of configuration in glycerol and loss of the C(2) hydrogen might be the result of an oxidation–reduction sequence and proposed a biosynthetic pathway in which dihydroxyacetone phosphate (DHAP), rather than GP, is alkylated by GGPP. The resulting ketoether is then reduced to (*S*)-GGGP. We now report studies with cell-free extracts from *M. thermoautotrophicum* and *H. halobium* which establish activities for isopentenyl diphosphate (IPP) isomerase, GGPP synthase, and GGGP synthase in both archaebacteria and show that (*S*)-GP is also the preferred acceptor for the prenyl transfer reaction catalyzed by GGGP synthase in *H. halobium*.

Experimental Section

Materials and General Methods. [^{14}C]IPP (37.6 μCi/μmol) was purchased from Amersham. DMAPP, GPP, FPP, GGPP, phytyl diphosphate, and phytanyl diphosphate were prepared as previously described.[17,25] Epoxyisopentenyl diphosphate

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(16) Abbreviations used: BHDA, bicyclo[2.2.1]cyclohept-5-ene-2,3-dicarboxylic acid; DTT, dithiothrietol; DHAP, dihydroxyacetone phosphate; DMAPP, dimethylallyl diphosphate; EBPP, epoxybutyl diphosphate; EIPP, 3,4-epoxy-3-methylbutenyl diphosphate; FPP, farnesyl diphosphate; GGGP, geranylgeranylglyceryl phosphate; GP, glyceryl phosphate; GPP, geranyl diphosphate; GGPP, geranylgeranyl diphosphate; IPP, isopentenyl diphosphate.
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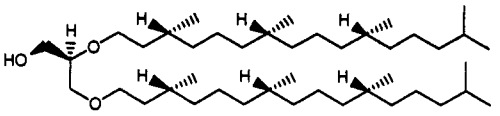
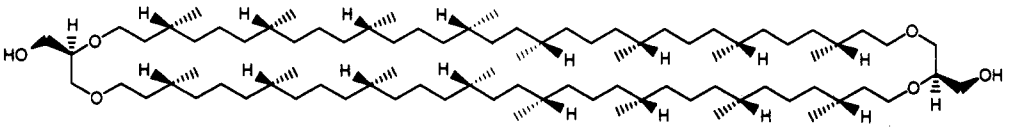
Chart I



C_{20} Diether



C_{40} Tetraether

(EIPP) was prepared by direct epoxydation of IPP by *m*-chloroperoxybenzoic acid,[26] and 3,4-epoxybutenyl diphosphate (EBPP) was prepared as described Muehlbacher and Poulter.[27] (*S*)-[1-^{3}H]Glyceryl phosphate (7 Ci/mol) was synthesized as described previously.[17] *H. halobium*, R1, was grown as described by Oesterhelt and Stoeckenius.[28] Frozen cells of *M. thermoautotrophicum*, Marburg, were provided by Professor Lacy Daniels. Other reagents were purchased from Sigma or Aldrich.

All buffers were prepared using deionized water. The pH of the buffers was adjusted with 3 M HCl or 3 M KOH. Radioactivity was determined by liquid scintillation spectrometry in 10 mL of Opti-fluor. Protein concentrations were determined as described by Spector.[29]

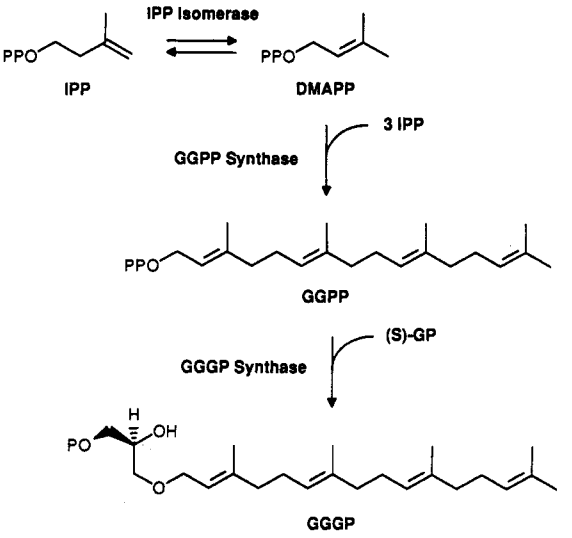
Cell-Free Studies. Cell-free extracts were prepared from 5 g of wet cells from *M. thermoautotrophicum* or *H. halobium*. Cells were suspended in 20 mL of buffer A (50 mM BHDA, 3 mM $MgCl_2$, 1 mM DTT, and 20 μM EDTA, pH 7.2) for *M. thermoautotrophicum* or buffer B (buffer A containing 4 M NaCl) for *H. halobium* and disrupted by sonication. Cellular debris was removed by centrifugation at 8000*g* for 15 min. The clarified extracts were further centrifuged at 200000*g* for 2 h at 4 °C to pellet microsomal proteins. The supernatants, which contained cytosolic proteins, were used immediately or stored −70 °C.

IPP Isomerase Assay. The assay was similar to the procedure described by Muehlbacher and Poulter.[27] A mixture of 100 μM [^{14}C]IPP (10 Ci/mol) in 100 μL of buffer A (*M. thermoautotrophicum*) or buffer B (*H. halobium*) was incubated at 37 °C for 10 min with cytosolic protein. The reaction was terminated by addition of 0.2 mL of methanol/concentrated HCl (4:1, v/v) and incubated for an additional 10 min. Ligroin (1 mL) was added, and the suspension was agitated on a vortex mixer for 5 s. Radioactivity was measured in a 0.5-mL portion of the ligroin layer.

GGPP Synthase Assay. The assay was similar to the procedure reported by Chan and Poulter.[30] A portion of the cytosolic protein was preincubated with 50 μM EBPP for 5 min at 37 °C to inactivate IPP isomerase.[26,27] Mixtures of 100 μM [^{14}C]IPP (10 Ci/mol) and 200 μM DMAPP, GPP, or FPP in 100 μL of buffer A (*M. thermoautotrophicum*) or buffer B (*H. halobium*) were incubated with pretreated cytosolic protein for 10 min at 37 °C. The reactions were terminated by addition of 0.2 mL of methanol/concentrated HCl (4:1 v/v). The samples were worked up and analyzed as described for the IPP isomerase assay.

GGGP Synthase Assay. The assay was similar to the procedure described by Zhang and Poulter.[17] A mixture of 150 μM (*S*)-[^{3}H]GP (3 Ci/mol) and 200 μM GGPP in 150 μL of buffer A (*M. thermoautotrophicum*) or buffer B (*H. halobium*) was incubated with cytosolic protein. The reaction was initiated by addition of GGPP, incubated at 37 °C for 10 min, and terminated by addition of 100 μL of 0.5 M EDTA to sequester Mg^{2+}. Saturated brine (1 mL) and water-saturated 1-butanol (3 mL) were added. After the mixture was vigorously mixed for 10 s, the butanol layer was allowed to separate, and the radioactivity in a 1-mL portion of the butanol extract was measured.

Scheme I



Results and Discussion

Activities for IPP Isomerase, GGPP Synthase, and GGGP Synthase. Activities for enzymes that activate the electrophilic isoprene unit (IPP isomerase), synthesize the C_{20} substrate for archaebacterial membrane lipids (GGPP synthase), and attach GGPP to (*S*)-GP (GGGP synthase) were established in cytosolic preparations of *M. thermoautotrophicum* and *H. halobium* (see Scheme I). Activity for IPP isomerase was identified by incubating cytosolic protein from the two bacteria with [^{14}C]IPP. The assay involves addition of acidic methanol to the incubation mixture. This destroys the enzyme and selectively decomposes DMAPP. Isomerization of homoallylic IPP to allylic DMAPP activates the hydrocarbon moiety toward solvolysis. At low pH where the diphosphate moiety is a better leaving group, DMAPP reacts to give a mixture of hydrocarbon, methyl ethers, and alcohols, all of which are soluble in organic solvents, whereas IPP is stable and remains in the aqueous layer. The amount of DMAPP produced is determined by measuring the amount of radioactivity extractable by ligroin. At low conversions of IPP, the amount of material carried forward in subsequent prenyl transfer steps (see below) is small and does not interfere with the assay. As seen in Table I, both archaebacteria have comparable levels of IPP isomerase.

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Table I. Activities of IPP:DMAPP Isomerase and GGPP Synthase in Cell-Free Extracts of *H. halobium* and *M. thermoautotrophicum*

enzymes	substrates	Sp Act[a] × 10^5 *H. halobium*	Sp Act[a] × 10^5 *M. thermoautotrophicum*
IPP isomerase	[^{14}C]IPP	4.6	4.6
GGPP synthase	[^{14}C]IPP + DMAPP	3.6	7.4
	[^{14}C]IPP + GPP	3.3	8.5
	[^{14}C]IPP + FPP	3.3	4.8
GGGP synthase	(S)-[^{3}H]GP + GGPP	11	39

[a] μmol min^{-1} mg^{-1}.

Table II. Substrate Selectivity of GGGP Synthase from *H. halobium*[a] and *M. thermoautotrophicum*[b]

acceptors[c]	donors[d]	Sp act[e] × 10^5 *H. halobium*	Sp act[e] × 10^5 *M. thermoautotrophicum*[f]
(*S*)-[^{3}H]GP	GGPP	11	39
(*R*)-[^{14}C]GP	GGPP	0.3	1
[^{14}C]GOH	GGPP	<0.1	<0.1
[^{32}P]DHAP	GGPP	<0.1	<0.1
(*S*)-[^{3}H]GP	phytyl-PP	2.5	7
(*S*)-[^{3}H]GP	phytanyl-PP	<0.1	<0.5

[a] Determined in 50 mM BHDA, 4 M NaCl, pH 7.2, at 37 °C. [b] Determined in 50 mM BHDA, pH 7.2, at 37 °C. [c] [150 μM]. [d] [200 μM]. [e] μmol min^{-1} mg^{-1}. [f] Reference 17.

The assay for GGPP synthase depends on conversion of [^{14}C]IPP to reactive allylic diphosphates (GPP, FPP, or GGPP) during chain elongation, which are also detected by the acid lability procedure. Interference from IPP isomerase in the chain elongation assay was eliminated by pretreating the cytosolic protein with EIPP or EBPP. These compounds are potent irreversible inhibitors of IPP isomerase from yeast[26] and Claviceps.[27] Treatment of cell-free extracts from *M. thermoautotrophicum* or *H. halobium* with 50 μM EIPP or EBPP rapidly inactivated IPP isomerase, and within 10 min, isomerase activity was reduced to less than 2% of uninhibited controls. Addition of IPP to the incubation decreased the rate of inactivation, presumably by competing with the epoxides for binding to IPP isomerase as expected for an active-site directed inhibitor.

We recently purified GGPP synthase from *M thermoautotrophicum* and discovered that the enzyme is a bifunctional protein which generates substantial pools of both FPP and GGPP for subsequent conversion to tri- and tetraterpenes, respectively.[30] Although the corresponding enzyme from *H. halobium* was not purified in this study, its activity was similar to GGPP synthase in cytosolic preparations from *M. thermoautotrophicum*. In particular, as shown in Table I, both enzymes utilized DMAPP, GPP, and FPP as allylic substrates with similar efficiencies.

The specific activities for GGGP synthase in *M. thermoautotrophicum* and *H. halobium* are given in Table I. In a separate experiment, the product from incubation of [^{3}H]GP and GGPP with cytosolic protein from *H. halobium* was treated with alkaline phosphatase and analyzed by reversed-phase HPLC.[17] The radioactivity comigrated with an authentic sample of geranylgeranylglycerol. These results are identical to those previously reported for *M. thermoautotrophicum*.[17]

In early experiments, very low levels of activity were seen for *H. halobium* GGGP synthase when the assays were conducted in low salt buffers. Since the organism thrives in saturated solutions of NaCl, we investigated the effect of NaCl on the stability of the enzyme. Cytosolic proteins from the extreme halophile were incubated in 50 mM BHDA buffer containing 0, 2.5, and 4.0 M NaCl. The samples were kept on ice and were assayed periodically for GGGP synthase activity. In salt-free buffer, the enzyme lost >90% of its activity in 2 h. The stability of GGGP synthase increased with increasing concentrations of NaCl. In 2.5 M salt, the enzyme retained half of its activity, and the activity was stable for up to 3 days in 4 M NaCl. The requirement for high-salt concentrations by *H. halobium* GGGP synthase is typical of other enzymes from extreme halophiles.[31-33]

Substrate Selectivity for *H. halobium* GGGP Synthase. (*S*)-[^{3}H]GP, (*R*)-[^{14}C]GP, [^{14}C]glycerol, and [^{32}P]-DHAP were individually incubated with GGPP and cytosolic GGGP synthase. As shown in Table II, (*S*)-GP was strongly preferred over other prenyl acceptors, including DHAP. In incubations with (*S*)-GP, GGPP was the preferred prenyl donor, although phytyl diphosphate was a moderately active alternate substrate. The completely saturated phytanyl derivative did not alkylate (*S*)-GP. The substrate selectivities seen for *H. halobium* GGGP synthase are very similar to those previously reported for the methanogenic enzyme.[17] It is clear that (*S*)-GP is the preferred prenyl acceptor, and an allylic isoprenoid diphosphate is required as a prenyl donor for both enzymes.

Biosynthetic Considerations. Early studies indicated that isoprenoid biosynthesis in archaebacteria followed the same mevalonate pathway found in eukaryotes.[34-37] There are, however, some interesting differences. For example, eukaryotes typically have two distinct enzymes for synthesis of FPP and GGPP.[38,39] However, we recently purified the enzyme responsible for chain elongation of DMAPP in *M. thermoautotrophicum*,[30] and it is clear that a single enzyme is responsible for synthesis of both FPP and GGPP. Although we have not purified the related enzyme in *H. halobium*, the similarities we found for GGPP synthase activities in cytosolic fractions from the two archaebacteria leave little doubt that the prenyltransferases for chain elongation of DMAPP are closely related.

The similarities between the halophilic and methanogenic GGGP synthases are also striking. The substrate selectivities for a variety of prenyl donors and prenyl acceptors are virtually identical when corrected for differences in the specific activities of the two enzymes in cytosolic preparations. The inability of GGGP synthase from *H. halobium* to utilize DHAP as a prenyl acceptor

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is strong evidence against the suggestion that inversion at C(2) of glycerol occurs via a geranylgeranyldihydroxyacetone ether.[23] While the C_3 portion of the pathway for biosynthesis of archaebacterial core membrane lipids remains cryptic, it is clear that the prenyl-transfer steps for chain elongation and alkylation of glycerol are identical in *M. thermoautotrophicum* and *H. halobium*.

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