(S)-2,3-Di-O-geranylgeranylglycerol Phosphate Synthase from the Thermoacidophilic Archaeon Sulfolobus solfataricus

MOLECULAR CLONING AND CHARACTERIZATION OF A MEMBRANE-INTRINSIC PRENYLTRANSFERASE INVOLVED IN THE BIOSYNTHESIS OF ARCHAEAL ETHER-LINKED MEMBRANE LIPIDS*

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Hisashi Hemmi‡, Kyohei Shibuya, Yoshihiro Takahashi, Toru Nakayama, and Tokuzo Nishino

From the Department of Biomolecular Engineering, Graduate School of Engineering, Tohoku University, Aoba-yama 07, Sendai, Miyagi 980-8579, Japan

The core structure of membrane lipids of archaea have some unique properties that permit archaea to be distinguished from the others, i.e. bacteria and eukaryotes. (S)-2,3-Di-O-geranylgeranylglycerol phosphate synthase, which catalyzes the transfer of a geranylgeranyl group from geranylgeranyl diphosphate to (S)-3-O-geranylgeranylglycerol phosphate, is involved in the biosynthesis of archaeal membrane lipids. Enzymes of the UbiA prenyltransferase family are known to catalyze the transfer of a prenyl group to various acceptors with hydrophobic ring structures in the biosynthesis of respiratory quinones, hemes, chlorophylls, vitamin E, and shikonin. The thermoacidophilic archaeon Sulfolobus solfataricus was found to encode three homologues of UbiA prenyltransferase in its genome. One of the homologues encoded by SS00583 was expressed in Escherichia coli, purified, and characterized. Radio-assay and mass spectrometry analysis data indicated that the enzyme specifically catalyzes the biosynthesis of (S)-2,3-di-O-geranylgeranylglycerol phosphate. The fact that the orthologues of the enzyme are encoded in almost all archaeal genomes clearly indicates the importance of their functions. A phylogenetic tree constructed using the amino acid sequences of some typical members of the UbiA prenyltransferase family and their homologues from S. solfataricus suggests that the two other S. solfataricus homologues, excluding the (S)-2,3-di-O-geranylgeranylglycerol phosphate synthase, are involved in the production of respiratory quinone and heme, respectively. We propose here that archaeal prenyltransferases involved in membrane lipid biosynthesis might be prototypes of the protein family and that archaea might have played an important role in the molecular evolution of prenyltransferases.

The structures of membrane lipids have some interesting and remarkable properties that enable us to distinguish archaea from other organisms, i.e. eukaryotes and bacteria (1) (Fig. 1). Although the archaeal “diether” membrane lipids are homologues of glycerolipids in other organisms, they differ with respect to the following features: 1) The hydrocarbon moieties of the archaeal lipids are fully reduced C20 or C25 prenyl groups, whereas the ordinary glycerolipids contain linear acyl groups. 2) The alkyl groups are attached to glycerol via an ether bond in archaeal lipids, while glycerol and the acyl chains are ester-bonded in the bacterial and eukaryotic glycerolipids. 3) The two groups of membrane lipids have opposite chiralities at their glycerol moieties; in short, the glycerol moieties of the archaeal and other glycerolipids are sn-2,3-di-O-alkylated and sn-1,2-di-O-acetylated, respectively. Moreover, the existence of circular “tetaether” lipids, which are synthesized from two molecules of diether lipids in methanogenic and thermophilic archaea, emphasizes the uniqueness of the archaeal membrane lipids.

The biosynthesis of the core structure of archaeal membrane lipids has been studied to date (Fig. 2). The genes of (all-E) geranylgeranyldiphosphate (GGPP)* synthase (GGPS), which catalyzes the production of the general precursor of the alkyl moieties of ordinary archaeal lipids, have been cloned from the thermophilic archaea, Sulfolobus acidocaldarius (2) and Archaeoglobus fulgidus (3), and a methanogenic archaea Methanobacterium thermoautotrophicum (4), and homologues have been identified in the genomes of various archaea. It is also noteworthy that some archaea such as halophilic Natronobacterium pharaonis (5) and thermophilic Aeropyrum pernix (6) utilize the longer farnesylgeranyl diphosphate as a precursor of alkyl groups. Nishihara et al. (7,8) reported that the formation of sn-glycerol-1-phosphate (G-1-P), the precursor of the glycerol moiety, from dihydroxy aceton phosphate, is catalyzed by G-1-P dehydrogenase in M. thermoautotrophicum. Zhang and Poultet (9–11) showed that the formation of ether linkages between G-1-P and two molecules of GGPP in M. thermoautotrophicum is catalyzed by distinct prenyltransferases. The first step, yielding (S)-3-O-geranylgeranylglycerol phosphate (GGGP), is catalyzed by GGGP synthase (GGPS). This enzyme was characterized in detail by Zhang et al. (12), and the GGPS genes were recently independently cloned from M. thermoautotrophicum (13) and Thermoplasma acidophilum (14). The second step that involves the production of (S)-2,3-di-O-geranylgeranylglycerol phosphate (DGGGP) from GGGP and GGPP is catalyzed by a microsomal enzyme, DGGGP synthase.

4 The abbreviations used are: GGPP, (all-E)-geranylgeranyldiphosphate; GGPS, GGPP synthase; G-1-P, sn-glycerol-1-phosphate; GGGP, (S)-3-O-geranylgeranylglycerol phosphate; GGGS, GGPP synthase; DGGGP, (S)-2,3-di-O-geranylgeranylglycerol phosphate; DGGS, DGGG synthase; PFP, (all-E)-farnesyl diphosphate; IPP, isopentenyl diphosphate; CHAPS, (3-[3-cholamidopropyl]dimethylammonio]-1-propanesulfonate; α-GP, α-glycerophosphate; HPLC, high-performance liquid chromatography; FAB-MS, fast atom bombardment-mass spectrometry; ORF, open reading frame; G-3-P, sn-glycerol-3-phosphate; HPT, homogentisate phytyltransferase. 4
Archaeol, respectively. Bacterial glycerolipid (B) and tetraether (A) lipids are shown. The structure of bacterial glycerolipid (C) is also represented for comparison. X represents polar head groups. When X is H, the archaeal lipids are denoted as archaeol and caldoarchaeol, respectively.

**Isolation of Archaeal Prenyltransferase Genes—** The ubiA-2 gene was amplified by means of a PCR by using primers specific to the 5′- and 3′-ends; 5′-AGCGTTATGGAGTTAAAATCTGATATG-3′ and 5′-TACGATGAGATCGGCTTAAAGAATGGAAG-3′, respectively. The genome of *S. solfataricus*, as a template, and KOD DNA polymerase (TOYOBO) were used in the reaction. The new restriction sites introduced into the primers, the NdeI and BamHI sites, are indicated with underlines. The amplified fragment, extracted from 0.8% agarose gel after electrophoresis, was digested with NdeI and BamHI and then ligated into the NdeI-BamHI sites of the pET-15b vector (Amersham Biosciences). The resultant plasmid was designated as pET-HisUbiA2. The *S. solfataricus* orthologue of GGPS gene was also amplified with the PCR primers, 5′-AGAGAACAATAGCTATCTAGAGGAAAGAAATG-3′ and 5′-CTTCTGGATCCCTTACATCTCCTATCCTAATCCC-3′, and the DNA fragment, after digestion with NdeI and BamHI, was ligated into the pET-15b vector to construct pET-HisGGPS. The *S. acidocaldarius* gds gene, encoding GGPS, was amplified with the primers, 5′-AGTACTTTGAGCTATGAGTTACCTGTITGACCAAATCTTTTTATTT3′ and 5′-TCCGAGATGGCTTTTCTTATCGTAAAT-3′. The amplified fragment was digested with NdeI and BamHI and ligated into pET-15b to construct pET-HisGGPS.

**Expression and Purification of the Recombinant Enzymes—** *E. coli* BL21(DE3) transformed with each of the plasmids, as described above, was cultivated in 1 liter of M9YG broth supplemented with ampicillin (50 mg/liter). When the *A*$_{soy}$ of the culture reached 0.5, the transformed bacteria were induced with 1.0 mM isopropyl-1-thio-β-D-galactoside. After an additional 5 h cultivation, the cells were harvested and disrupted by sonication in HisTrap (Amersham Biosciences) binding buffer, containing 20 mM potassium phosphate, pH 7.6, 0.5 mM NaCl, and 10 mM imidazole (100 mM for the purification of UbiA-2), prepared following the manufacturer’s instructions. The homogenate was centrifuged at 4,000 × g for 15 min, and the supernatant was recovered as a crude extract. The crude extract was heated at 55 °C for 1 h, and the denatured proteins were removed by centrifugation at 6,000 × g for 20 min. Note that in the case of the purification of UbiA-2 protein, CHAPS (10 mM) was added to the crude extract to solubilize the recombinant protein before the heat treatment. The supernatant fraction was loaded on a HisTrap column, which had been equilibrated with the HisTrap binding buffer. The column was washed with the binding buffer, and proteins that had specifically bound were then eluted with a HisTrap elution buffer, containing 20 mM potassium phosphate buffer, pH 7.6, 0.5 mM NaCl, and 350 mM imidazole, and used for characterization. The level of purification was determined by 12.5% SDS-PAGE.

**Radio-assays for Prenyltransferase Reactions—** The assay mixture contained, in a final volume of 200 μl, 0.5 nmol of [1-14C]IPP (2.04 GBq/mmol), 0.5 nmol of (all-3′-F)FPP, 0.2 μg of α-glycerophosphate (α-GP), 2 μM of MgCl$_2$, 10 μM of sodium phosphate buffer, pH 5.8, and suitable amounts of enzymes, i.e. GGPS, GGGPS, and/or UbiA-2. These mixtures were incubated at 55 °C for 1 h, and the reaction was stopped by adding 200 μl of a cold, saturated solution of NaCl. The mixture was then chilled with 600 μl of 1-butanol saturated with H$_2$O. The hydrophobic products extracted with 1-butanol were treated with acid phosphatase according to the method of Fuji et al. (19) The hydrolysates were extracted with pentane and analyzed by reversed-phase TLC using a precoated plate, LKC-18F, developed with ace tone/H$_2$O (9:1). Authentic standard geranyleraninal was visualized by exposure of the plate to iodine vapor, and the distribution of radioactivity was detected using a molecular imager (Bio-Rad).

**Experimental Procedures**

**Materials—** Precoated reversed-phase thin-layer chromatography plates, LKC-18F were purchased from Whatman Chemical Separation, Inc. GGPP and (all-3′-F)farnesyl diphosphate (FPP) were donated by Drs.

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Enzymatic Preparation of GGGP—To synthesize radiolabeled GGGP, the same reaction mixture as was used in the radio-assay was employed, although UbiA-2 was removed. Non-labeled GGGP was produced by replacing only GGPP, replacing with [3H]GGPP and [3H]IPP by GGPP. The butanol-extracted compounds were concentrated and loaded onto a YMC-Pack ODS-A column (4.6 × 250 mm, YMC) connected to a high performance liquid chromatograph (HPLC) system. They were eluted with eluant A (25 mM NH4HCO3) isocratically for the first 5 min and then with a linear gradient from 100% eluant A to 100% eluant B (acetonitrile) through 30 min, and finally with eluant B for 25 min, at a flow rate of 0.5 ml/min, under the method of Zhang and Poulter (20), with minor modifications. The eluted products were detected by UV absorption at 210 nm or by radioactivity measured with a ramona Star radio-HPLC analyzer (Raytest). GGGP was eluted at about 34 min, whereas GGPP emerged at about 30 min. A fast atom bombardment-mass spectrometry (FAB-MS) analysis of non-labeled GGGP was performed with an MStation JMS-700 mass spectrometry system (JEOL) in the negative ion mode, using diethanolamine as the matrix. Analysis of the Product of Prenyltransferase Reaction Catalyzed by UbiA-2—The reaction mixture contained, in a final volume of 400 μl, 120 nmol of GGGP, 200 nmol of α-GP, 4 μmol of MgCl2, 20 μmol of sodium phosphate buffer, pH 5.8, and suitable amounts of GGPPS and UbiA-2. This mixture was incubated at 55 ℃ for 1 h, and hydrophobic compounds were extracted with 1.2 ml of 1-butanol. The butanol layer was concentrated and loaded on a COSMOSIL SC3-A,AR-300 column (4.6 × 150 mm, Nacalai Tesque). The compounds were eluted from the column with the same eluant profile as was used in the HPLC analysis of GGGP formation described above. Elution of the compounds was detected by UV absorption at 210 nm. In addition to the absorption peak of GGGP, which eluted at about 27 min, a peak eluting at about 35 min was observed. An FAB-MS analysis of the product that co-eluted with the peak produced with an MStation JMS-700 mass spectrometry system (JEOL) in the negative ion mode, using diethanolamine as the matrix. In addition, the product was treated with acid phosphatase by a method described by Fujii et al. (19), extracted with pentane, and analyzed by FAB-MS in the positive ion-mode. A double-labeling experiment was conducted using an assay mixture containing, in a final volume of 200 μl, 1 nmol of [methyl-3H]GGPP, 2 nmol of [3H]IPP, 20 nmol of [14C]IPP, 80 nmol of α-GP, 4 μmol of MgCl2, 10 μmol of sodium phosphate buffer, pH 5.8, and a suitable amount of UbiA-2. The reaction product was hydrolyzed with acid phosphatase and analyzed by HPLC, using a YMC-Pack ODS-A column (4.6 × 250 mm, YMC) eluted with methanol/2-propanol (7:3). The proportion of [3H] and [14C] radioactivities that co-eluted with the peak of the new product was measured using a ramona Star radio-HPLC analyzer (Raytest).

The specificity of UbiA-2 for prenyl donor substrates was examined by replacing the [3H]GGPP with 60 nmol of GGPP, (all-E)FPY, or phytyl diphosphate in the reaction mixture for the double-labeling experiment. To investigate the specificity for prenyl acceptor substrates, [14C]GGPP was replaced with α-GP, 4-hydroxybenzoate, 1,4-dihydroxy 2-naphthoate, or homogentisate. The formation of reaction products was confirmed by HPLC using the same method as described above.

Phylogenetic Analysis—Amino acid sequences of some typical enzymes classified as members of the "UbiA prenyltransferase family" according to the Pfam protein family data base (www.sanger.ac.uk/Software/Pfam/) were obtained from other public protein databases. They were aligned using the ClustalW 1.8 program on the DDBJ web site (spiral.genes.nig.ac.jp/homology/welcome-e.shtml), and the phylogenetic tree was constructed with the neighbor-joining method. All parameters used in the program were at the default settings.

RESULTS

Isolation of a Novel, Hypothetical Prenyltransferase Gene of S. solfataricus—We noted that, in the entire genome sequence of S. solfataricus, there are two open reading frames (ORFs) encoding homologues of UbiA, a well known prenyltransferase for ubiquinone biosynthesis. The ORFs, SS00125 and SS00583, are designated as ubia-1 and ubia-2, respectively. Because S. solfataricus is known to produce caldariellaquinone, not ubiquinone, as an electron carrier compound in its respiratory chain, one of the two ORFs is thought to encode the enzyme involved in the biosynthesis of the respiratory quinone. The UbiA-1 protein from S. solfataricus has a higher similarity (25.9% identity) with E. coli UbiA octaprenyl diphosphate:4-hydroxybenzoate octaprenyltransferase than S. solfataricus UbiA-2 (20.1% identity). Indeed, the result of an orthologue analysis using the www server of the Microbial Genome Data Base (mbgd.genome.ad.jp/) indicated that UbiA-1 is an orthologue of E. coli UbiA, suggesting that the enzyme is likely the prenyltransferase involved in caldariellaquinone biosynthesis. On the other hand, UbiA-2 was treated in the data base as an orthologue of cyanobacterial chlorophyll a synthase ChlG, which is also homologous with UbiA prenyltransferase and catalyzes the transfer of a phytol group to chlorophyllide a. Moreover, almost all archaea whose entire genome sequences have been determined, except for Nanoarchaeum equitans, appeared to possess the predicted orthologues of UbiA-2. Because it is generally thought that archaea do not require either chlorophyll or bacteriochlorophyll, we initially thought that UbiA-2 might be involved in the biosynthesis of prevylated hemes, which have the porphyrin structure of chlorophyll. However, we immediately noted that the genome of S. solfataricus also encodes a probable orthologue of ferroso protoheme IX farne-syltransferase, in SS00656, another ORF homologous to ubia. These facts strongly suggest that UbiA-2 and its predicted orthologues encoded in the archaeal genomes have an unrevealed, but very important function in archaeal cells. The archaeal lipid-biosynthetic reaction in which a geranylgeranyl group is transferred to GGGP, an acyclic prenyl acceptor, to produce DGGGP was a promising candidate for the function, although the other reactions catalyzed by the homologues of UbiA studied thus far typically require acceptors that contain aromatic or porphyrin structures. It should be noted here that the symbiotic archaean Nanoarchaeum equitans is known to lack lipogenic genes in its genome (21), so it seems reasonable that it should have no DGGGPS gene. Thus, we decided to isolate the ORF SS00583, encoding a protein of 282 amino acids, to characterize its function. The ORF was amplified by PCR from the genome of S. solfataricus, and the amplified DNA fragment was digested with restriction enzymes and inserted into pET15b, an expression vector for E. coli.

Expression and Purification of Recombinant S. solfataricus UbiA-2—The recombinant UbiA-2 protein with a hexameric histidine tag at its N terminus was expressed in the cells of E. coli. After heat treatment of a crude extract from E. coli cells, the recombinant protein was precipitated without any loss of activity, probably because it is associated with the membrane of the host. However, by adding 0.2% CHAPS to the crude extract, we succeeded in solubilizing the enzyme, which enabled us to purify the enzyme by removing proteins from the host by means of a

**FIG. 3. SDS-PAGE analysis of recombinant UbiA-2 protein.** Lane 1, molecular standard marker; lane 2, crude extract from E. coli BL21(DE3)/pET15b; lane 3, crude extract from E. coli BL21(DE3)/pET15b-UbiA2; lane 4, supernatant fraction after the heat treatment of the crude extract from E. coli BL21(DE3)/pET15b-UbiA2; lane 5, flow-through fraction from a nickel-chelating affinity column; lane 6, recombinant UbiA-2 purified with a nickel-chelating affinity column chromatography.

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heat treatment at 55 °C for 1 h. We then used metal chelating affinity column chromatography for further purification of the UbiA-2 protein. As shown in Fig. 3, a major protein band was observed by SDS-PAGE analysis. Although the molecular mass of histidine-tagged UbiA-2, estimated from the SDS-PAGE analysis to be 28 kDa, was slightly smaller than that calculated from its amino acid sequence, this situation might arise from the high thermostability of the enzyme and/or the fact that the enzyme is membrane intrinsic.

Enzymatic Synthesis of GGGP—To synthesize substrates for the DGGGPS reaction, two prenyltransferases that catalyze former reactions, i.e., GGPS and GGGPS, were prepared. The S. acidocaldarius GGPS gene *gds*, which we had cloned previously (2), and the S. solfataricus putative GGGPS gene SSO0259, which was assigned based on its high homology with known GGGPSs and amplified by PCR, were inserted into an expression vector pET15b, respectively. Both prenyltransferases were efficiently expressed as histidine-tagged proteins in *E. coli* cells and were purified from the crude extracts by a heat treatment and metal affinity chromatography. We were able to produce radiolabeled GGPP from [14C]IPP and FPP, which could be confirmed by the TLC analysis of the product extracted with 1-butanol, after hydrolysis with acid phosphatase (Fig. 4A, lane 1). By adding purified GGPP to the reaction mixture for GGPS, a new radiolabeled spot was detected by TLC analysis using the same procedure (Fig. 4A, lane 2). These reaction mixtures contained G-1-P, a mixture of G-1-P and sn-glycerol-3-phosphate (G-3-P), as the prenyl acceptor substrate for GGGPS, because G-1-P, which is the substrate for GGGPSs from *M. thermoautotrophicum* and *T. acidophilum*, was not commercially available. However, the actual substrate for the predicted GGGPS is considered to be G-1-P as well, because the reaction did not proceed when commercial G-3-P was used (data not shown). The peak of a new product was found, in addition to the GGPP peak, in the reversed-phase HPLC analysis of hydrophobic compounds extracted from the incubated reaction mixture containing GGPS, GGGPS, and non-labeled substrates. The new product was confirmed to be Digeranylgeranylglyceryl Phosphate Synthase.
GGGP by negative-mode FAB-MS: an ion peak with an m/z value of 443, which corresponds to [M-H]⁻ of GGGP, was found. The enzymatically synthesized GGGPS, which could be produced in either non-labeled or radiolabeled forms, was used for the characterization of UbiA-2.

**Function of UbiA-2 Protein**—By the addition of the UbiA-2 protein to the reaction mixture for GGGPS, which contains GGGP and GGGP, the generation of a new radiolabeled spot was observed by the TLC analysis of the phosphatase-treated products (Fig. 4A, lane 3). The new spot disappeared, and only the spot of geranylgeraniol was observed, when GGGPS was removed from the mixture (Fig. 4A, lane 4). Using the purified UbiA-2 protein and the substrates, GGGP and the enzymatically synthesized GGGPS, we attempted to examine the activity of UbiA-2 as DGGGPS. By a HPLC analysis using a C4 reversed-phase column, a new product was found. This product eluted from the column later than GGGP and was formed after incubation (Fig. 4B). The product was shown to give an ion peak with an m/z value of 716, which corresponds to [M-H]⁻ of DGGGPS, by negative FAB-MS (Fig. 4C). By the acid-phosphatase treatment of the product, we were able to obtain a pentane-extractable compound whose m/z value, as determined by positive FAB-MS analysis, was 637, which corresponds to the alcohol produced by the hydrolytic removal of a phosphate group from DGGGPS (Fig. 4D). Moreover, a double labeling experiment using [3H]GGGP and [14C]GGGP indicated that the ratio of these substrates used to form the product from the reaction of UbiA-2 was 1:1.10 ± 0.27. Collectively, these data strongly suggest that the product is DGGGPS. When GGGPS was substituted with other prenyl donors, i.e., FPP and phytol diphosphate, the formation of new products, which could have been synthesized by the prenyl transfer reaction with [14C]GGGP, was not found. On the other hand, 4-hydroxybenzocate, 1,4-dihydroxy 2-naphthoate, homogentisate, and α-GP did not function as prenyl acceptor substrates, at least when [3H]GGGP was used as the prenyl donor. These results clearly indicate that the reaction catalyzed by UbiA-2 is highly specific and that the archaeal enzyme is DGGGPS. The enzyme activity of DGGGPS disappeared when 10 mM Mg²⁺ was replaced with an equivalent concentration of EDTA, which indicates the requirement of a divalent metal ion for activity. The metal ion could be replaced by 10 mM Ca²⁺, although the enzyme activity fell by about 60%, whereas 10 mM Mn²⁺ and Zn²⁺ led to a severely decreased activity. 1 mM Mg²⁺ or Mn²⁺ gave about 40% of the activity observed with 10 mM Mg²⁺, whereas 1 mM Ca²⁺ and Zn²⁺ hardly activated the enzyme. The pH optimum for the enzyme reaction was shown to be 6.0.

**Structure of DGGGPS**—To determine the structural property of S. solfataricus DGGGPS, we compared its amino acid sequence with those of its orthologues encoded in the genomes of various archaea, i.e., thermophilic, methanogen, and halophilic archaea, and other prenyltransferases homologous with DGGGPS, such as E. coli UbiA 4-hydroxybenzocate octaprenyltransferase, E. coli MenA 1,4-dihydroxy 2-naphthoate octaprenyltransferase, Arabidopsis thaliana homogenisate phytlytransferase (HPT), Synechococcus ChlG chlorophyllide a phytlytransferase, and E. coli CyoE protoheme IX farnesyltransferase. As a result, these enzymes, all classified in the “UbiA prenyltransferase family” according to the Pfam protein family data base, contain a highly conserved region (Fig. 5). The corresponding region in E. coli UbiA and COQ2, the UbiA orthologue of Saccharomyces cerevisiae, was predicted to be the prenyl diphosphate-binding site, suggesting that archaeal DGGGPSs catalyze prenyl transfer reactions via the same mechanism as the other UbiA-related prenyltransferases. Moreover, all the enzymes classified in this family are considered to be, and some have been shown (22, 23), to be membrane-intrinsic, unlike other prenyltransferases such as GGPS and GGGPS. In fact, the hydrophobicity plot of S. solfataricus DGGGPS was very similar with that of E. coli UbiA, and they clearly show that the enzymes contain multiple transmembrane regions (data not shown).

**Phylogenetic Analysis of Membrane-Intrinsic Prenyltransferases**—To clarify the evolutionary relationships between DGGGPS and other members of UbiA prenyltransferase family, a phylogenetic tree was constructed with the amino acid sequences of S. solfataricus DGGGPS, its 5 orthologues from various archaea, 11 typical members of the UbiA prenyltransferase family, and 2 orthologues of such prenyltransferases from S. solfataricus, encoded in SSO0125 and SSO00656 (Fig. 6). In this tree, the existence of six clusters can be found, in which enzymes catalyzing similar reactions are included, i.e., the clusters of DGGGPS (light blue area), UbiA/COQ2 (orange area), CyoE/COX10 (pink area), ChlG/BchG (green area), HPT (purple area), and MenA (yellow area). A bootstrap analysis indicated that all of the clusters are apparently independent from each other. However, unlike the other clusters, the cluster of archaeal DGGGPS orthologues appears to be located near the center of the tree, from which the other clusters branch out distinctly. The two S. solfataricus homologues of UbiA-family prenyltransferases other than DGGGPS, SSO0125 (UbiA-1) and SSO00656, were located near the clusters of UbiA/COQ2 prenyltransferases for heme biosynthesis, ubiquinone (and shikonin) and CyoE/COX10 prenyltransferases for heme biosynthesis, respectively.

**DISCUSSION**

The UbiA prenyltransferase family of enzymes typically catalyze the transfer of a prenyl (or phytol) group to hydrophobic acceptors. However, the structure of the acceptor molecules varies broadly. Examples include prenyl groups attached to a carbon atom in the aromatic ring of the precursors of quinones or tocopherol, a carboxyl oxygen in a propionate side chain of chlorophyllides, a carbon in an ethylene side chain of protoheme, and an oxygen in the glycerol moiety of GGGP. It should be emphasized here that DGGGPS from S. solfataricus is the first example of a UbiA family prenyltransferase that utilizes an acyclic prenyl acceptor substrate. The phylogenetic tree of the protein family clearly has several clusters of enzymes, and the clustered enzymes accept prenyl-acceptors, not donors, with similar structures. For example, geranyl diphosphate-4-
The clustering of the UbiA prenyltransferase family. The colored areas represent the six clusters of subfamilies, i.e., DGGGPS and its archaeal orthologues (light blue), UbiA/COQ2 (orange), Cyto/COX10 (pink), ChlG/BchG (green), HPT (purple), and MenA (yellow). The paler-colored areas surrounding the UbiA/COQ2 and Cyto/COX10 clusters indicate larger ranges of subfamilies, including S. solfataricus orthologues whose functions are unidentified. Abbreviations and accession numbers of each protein are as follows: SynChlG, chlorophyll a synthase ChlG from Synechocystis sp. strain PCC 6803 (BAA10281); RbcChG, bacteriochlorophyll synthase subunit BchG from Rhodobacter capsulatus (CAA77532); SynHPT, homogentisate phytyltransferase Slr1736 from Synechocystis sp. strain PCC 6803 (BAA17774); AthPT1, homogentisate phytyltransferase HPT1 from A. thaliana (AAM10489); BcMenA, 1,4-dihydroxy 2-naphtoate polyprenyltransferase MenA from Bacillus cereus (AAP11757); EcMenA, 1,4-dihydroxy 2-naphtoate octaprenyltransferase MenA from E. coli (AAC76912); APE0159, a hypothetical protein from Aeropyrum pernix, annotated as the putative bacteriochlorophyll synthase (BAA79070); HalHhoA, HhoA protein from Halobacterium sp. NRC-1, annotated as the 4-hydroxybenzoate octaprenyltransferase (AAG19118); TA0696, a hypothetical protein from Thermoplasma acidophilum, annotated as the predicted 4-hydroxybenzoate polyprenyltransferase (NP_394456); MjUbiA, UbiA protein from M. jannaschii, annotated as the 4-hydroxybenzoate octaprenyltransferase (AAB98267); SsUbiA2, DGGGPS from S. solfataricus, named UbiA-2 (AAK40896); PH0027, a hypothetical protein from P. kurokohii OT3 (BAA29996); SsUbiA1, UbiA-1 protein from S. solfataricus, annotated as the 4-hydroxybenzoate octaprenyltransferase (AAK40480); EcUbiA, 4-hydroxybenzoate octaprenyltransferase UbiA from E. coli (AAC43134); LePGT1, 4-hydroxybenzoate geranylgeranylglycerol phosphate synthase PGT-1 from Lithospermum erythrorhizon (BAB84122); ScCOQ2, 4-hydroxybenzoate hexaprenyltransferase COQ2 from S. cerevisiae (CAA96321); SSO0656, hypothetical protein from S. solfataricus, annotated as the cytochrome c oxidase folding protein (AAK40961); ECyOE, protoheme IX farnesyltransferase CyOE from E. coli (AAC73551); and ScCOX10, protoheme IX farnesyltransferase COX10 from S. cerevisiae (CAA97879).

Hydroxybenzoate geranylglycerol Phosphate Synthase

Hydroxybenzoate geranylglycerol synthase from Lithospermum erythrorhizon, which is involved in shikonin biosynthesis, is included in the cluster of ubiquinone-biosynthetic UbiA/COQ2 polyisoprenyltransferases, which commonly accept 4-hydroxybenzoate. Considering the difference in the atoms to which the prenyl group is attached, it seems to be reasonable that the branches of chlorophyll- and heme-biosynthesizing enzymes are separated even though the structures of their prenyl-acceptor substrates, having a porphyrin ring, resemble each other. These facts indicate that the position of each enzyme in the tree clearly reflects its specificity for a prenyl-acceptor substrate. Therefore, the clustering of S. solfataricus DGGGPS and its probable orthologues from various archaea strongly suggests that they catalyze the same reaction.

As the result of a homology search of the entire genome sequence, S. solfataricus appeared to possess two UbiA family polyisoprenyltransferases, in addition to DGGGPS, whereas their functions are currently unknown. One, which shows a high similarity with 4-hydroxybenzoate polyisoprenyltransferases and is referred to as UbiA-1, is in close proximity to the UbiA/COQ2 proteins in the phylogenetic tree. Because the archaeon is known to use an anomalous respiratory quinone, caldariellaquinone, as a part of its respiratory system (24), the prenyltransferase would be expected to be involved in the biosynthesis of caldariellaquinone. The other is located near the CyOE/COX10 farnesyltransferases for heme biosynthesis, which suggests that this prenyltransferase catalyzes the biosynthesis of heme-like compounds. Indeed, the closely related S. acidocaldarius was reported to produce heme $A_2$, the geranylergynylated, not farnesylated, analogue of heme $A_2$. Through the data base search, we found that the genomes of archaea generally encode three to four proteins of the UbiA prenyltransferase family. However, the genomes of Pyrococcus sp., Methanopyrus kandleri, and Methanococcus jannaschii encode only one homologue with a high similarity to DGGGPS, which suggests the absence of prenylated hemes and respiratory quinones in these archaea. Prenylated quinones, which were frequently used as an index for the classification of microorganisms, have not been found from the organisms as far as we are aware. In addition, the genomes of all of these archaea contain only one gene encoding the homologue of prenyl diphosphate synthase, which is
respiratory quinones with unusual structures, cells, accompanying the duplication and subsequent evolution quinones could have also come into existence first in archaeal although the possibility of lateral transfer of prenyltransferase the role as the evolutional origin of all enzymes in this family, even imagine a scenario in which the archaeal enzyme played might be supported by the early divergences observed among history of DGGGPS could be considered to be as long as that of domain Archaea. The long evolutionary history of DGGGPS might be supported by the early divergences observed among enzymes included in the DGGGPS cluster. Therefore, we can even imagine a scenario in which the archael enzyme played the role as the evolutional origin of all enzymes in this family, although the possibility of lateral transfer of prenyltransferase genes from other organisms to archaea cannot be ignored. In this scenario, some prenylated compounds such as respiratory quinones could have also come into existence first in archael cells, accompanying the duplication and subsequent evolution of prenyltransferases. In fact, several archael species possess respiratory quinones with unusual structures, e.g. caldariella-quinone, thermoplasmaquinone, and sulfolobusquinone, whereas many thermophilic and halophilic archael produce menaquinones (26). Such unusual prenylquinones and meth-anophenazine (27, 28), a prenylated electron carrier compound that was recently discovered in a methanogenic archael **Methanosaarcina mazei** G01, which is known to have no prenylated quione, might be the traces of the molecular evolution of membrane-bound electron carrier compounds in archael. We have speculated as to why respiratory quinones, hemes, and chlorophylls contain prenyl groups as their hydrophobic side chains, whereas organisms other than archael are also able to synthesize linear acyl groups. The above scenario answers at least a part of the question. Archael seem to depend on isoprenoids more strongly than the other organisms, because almost all the lipid compounds in archael, e.g. membrane lipids, carotenoids, and respiratory quinones, are isoprenoids. Through the biological evolutional history, the roles that archael have played in the generation of isoprenoid biosynthetic enzymes and, moreover, isoprenoid compounds themselves might be greater than previously expected. Archael might be, so to speak, a cradle for isoprenoid biosynthesis.

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(S)-2,3-Di-O-geranylgeranylglyceryl Phosphate Synthase from the Thermoacidophilic Archaeon *Sulfolobus solfataricus*: MOLECULAR CLONING AND CHARACTERIZATION OF A MEMBRANE-INTRINSIC PRENYLTRANSFERASE INVOLVED IN THE BIOSYNTHESIS OF ARCHAEAL ETHER-LINKED MEMBRANE LIPIDS

Hisashi Hemmi, Kyohei Shibuya, Yoshihiro Takahashi, Toru Nakayama and Tokuzo Nishino

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