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Substrate specificity of undecaprenyl diphosphate synthase from the hyperthermophilic archaeon *Aeropyrum pernix*

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ABSTRACT

Cis-prenyltransferase from a hyperthermophilic archaeon *Aeropyrum pernix* was expressed in *Escherichia coli* and purified for characterization. Properties such as substrate specificity, product chain-length, thermal stability and cofactor requirement were investigated using the recombinant enzyme. In particular, the substrate specificity of the enzyme attracts interest because only dimethylallyl diphosphate and geranylfarnesyl diphosphate, both of which are unusual substrates for known *cis*-prenyltransferases, are likely available as an allylic primer substrate in *A. pernix*. From the enzymatic study, the archaeal enzyme was shown to be undecaprenyl diphosphate synthase that has anomalous substrate specificity, which results in a preference for geranylfarnesyl diphosphate. This means that the product of the enzyme, which is probably used as the precursor of the glycosyl carrier lipid, would have an undiscovered structure.

1. Introduction

Cis-prenyltransferase catalyzes consecutive *Z*-condensation of C_5 isoprene units to form (*Z*,*E*-mixed) prenyl diphosphate from an allylic primer substrate, i.e., (all-*E*) prenyl diphosphate, and multiple molecules of isopentenyl diphosphate (IPP) [1,2]. In general, bacterial and archaeal enzymes yield shorter products such as C_{55} undecaprenyl diphosphate (UPP), while eukaryotic enzymes give longer products. The products are used as the precursor of glycosyl carrier lipids, i.e., polyprenol for bacteria and dolichol for eukaryotes [3]. Specific dolichols, in which one or several double bonds at the ω-end are reduced, are reportedly used in halophilic and thermoacidophilic archaea [4–6], while methanogenic archaea use polyprenol or both polyprenol and dolichol [7–10]. The primer substrate generally used by the enzymes is C_{15} (all-*E*) farnesyl diphosphate (FPP) or C_{20} (all-*E*) geranylgeranyl diphosphate (GGPP), which yield products with 2 and 3 (*E*)-double bonds, respectively. Such short-chain (all-*E*) prenyl diphosphates are supplied by the action of *trans*-prenyltransferase such as FPP synthase and GGPP synthase. Shorter substrates are usually unfavorable for *cis*-prenyltransferases, with a few exceptions of the enzymes yielding very short products: bacterial (*Z*,*E*)-FPP synthase utilizes C_{10} geranyl diphosphate (GPP) as the primer substrate [11–14], and neryl diphosphate synthase [15] and (*Z*,*E*)-FPP synthase [16] from tomato catalyze one and two times of (*Z*)-condensation of IPP to C_5 dimethylallyl diphosphate (DMAPP), respectively.

In the genome of a hyperthermophilic archaeon *Aeropyrum pernix*, there is only one gene of (all-*E*) prenyl diphosphate synthase, which has been shown to encode geranylfarnesyl diphosphate (GFPP, also called farnesylgeranyl diphosphate) synthase [17]. This rare enzyme supplies a precursor of the C_{25} isoprenoid hydrocarbon chains of *A. pernix*-specific C_{25}–C_{25} diether-type membrane lipids. A gene encoding the homologue of *cis*-prenyltransferase is also found in the genome [18], implying that the enzyme is involved in the biosynthesis of the glycosyl carrier lipid of the hyperthermophile. However, this situation, with one (*Z*,*E*-mixed) and one (all-*E*) prenyl diphosphate synthase, suggests that the substrate of the *cis*-prenyltransferase is likely GFPP, which is the product of GFPP synthase, or DMAPP, which may be shared by GFPP synthase. The physiological role of the *cis*-prenyltransferase from *A. pernix* is unclear, but DMAPP is an unfavorable substrate for known *cis*-prenyltransferases involved in glycosyl carrier lipid biosynthesis [19–22]. Although the preference of *cis*-prenyltransferases toward GFPP has been rarely tested, UPP synthase from *Bacillus subtilis* reportedly accepts (all-*E*) GFPP, but about 5-times less intensely than the more-favored substrates (all-*E*) FPP and (all-*E*) GGPP [22]. Therefore, whichever substrate the enzyme from *A. pernix* utilizes, the product, i.e., (*Z*,*E*-mixed) prenyl diphosphate, and the resultant glycosyl carrier lipid are both expected to have structures that have never been reported.

Abbreviations: DMAPP, dimethylallyl diphosphate; FPP, farnesyl diphosphate; GGPP, geranylgeranyl diphosphate; GFPP, geranylfarnesyl diphosphate; GPP, geranyl diphosphate; HexPP, hexaprenyl diphosphate; IPP, isopentenyl diphosphate; UPP, undecaprenyl diphosphate.

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In the present study, *cis*-prenyltransferase from *A. pernix* was recombinantly expressed in *Escherichia coli* and purified for detailed characterization. Product determination of *A. pernix* GFPP synthase was also performed, and the data from the properties of the two prenyltransferases allowed us to infer an unusual biosynthetic pathway for the isoprenoid compounds in the hyperthermophilic archaeon.

2. Materials and methods

2.1. Materials

Precoated reversed-phase TLC plates LKC-18F and RP18 were purchased from GE healthcare, USA, and Merck Millipore, Germany, respectively. DMAPP, (all-*E*) FPP, and non-labeled IPP were donated by Dr. Chikara Ohto, Toyota Motor Co., Japan. [1-^{14}C]IPP was purchased from GE healthcare, USA. GPP was purchased from Sigma–Aldrich, USA. (All-*E*) GGPP was purchased from Larodan fine chemicals, Sweden. Polyprenol (mixture of C_{55} and C_{60} alcohol) was donated from Prof. Tokuzo Nishino, Tohoku University. All other chemicals were of analytical grade.

2.2. Cultivation of the microorganism

A. pernix, which was provided by the RIKEN BRC through the Natural Bio-Resource Project of the MEXT, Japan, was cultured in a JXT medium supplemented with 4 mM $Na_2S_2O_3{\cdot}5H_2O$ at 85 °C.

2.3. Cloning, expression and purification of A. pernix cis-prenyltransferase

From the cells of *A. pernix* harvested by centrifugation, the genome was extracted using an ISOPLANT II DNA extraction kit (Wako, Japan). The gene of *cis*-prenyltransferase homologue *ape_1385* was amplified by PCR using the genome of *A. pernix* as a template and primers 5′-TT*GGATCC*ATGGGACAGCCCAGAGCG-3′ and 5′-ATC*CTCGAG*TTACCTACCCATGGGCCTCC-3′. The newly introduced restriction sites for *Bam*HI and *Xho*I in the amplified gene (italicized in the primer sequences) were excised and then ligated into a pET-32a vector (Novagen, USA) to construct pET-32a-*ape_1385*. The plasmid was introduced into *E. coli* BL21 (DE3), and the transformant was grown at 37 °C in 200 mL LB medium, supplemented with 100 mg/L ampicillin. When the optical density at 600 nm of the culture reached 0.4, 1.0 mM IPTG was added for induction. After additional overnight cultivation, the cells were harvested and disrupted by sonication in a HisTrap binding buffer (20 mM sodium phosphate, 0.5 M NaCl, 20 mM imidazole, pH 7.4) supplemented with 16 mM CHAPS. The homogenates were centrifuged at 24,000*g* for 30 min to recover the supernatants as a crude extract. The crude extract was heated at 55 °C for 1 h to inactivate enzymes derived from *E. coli*. After centrifugation at 24,000*g* for 30 min, the supernatant was recovered. The heat-treated enzyme solution was loaded on a 1 mL HisTrap crude FF (GE healthcare, USA) column. The column was washed with 10 mL HisTrap binding buffer, and again with the same volume of the HisTrap binding buffer wherein the imidazole concentration was increased to 80 mM. The recombinant enzyme was eluted from the column with a HisTrap elution buffer (20 mM sodium phosphate, 0.5 M NaCl, 500 mM imidazole, pH 7.4). The level of purification was confirmed by 12% SDS–PAGE.

Cloning, expression and purification of A. pernix GFPP synthase – *A. pernix* GFPP synthase gene [17] was amplified by PCR using the genome of *A. pernix* as a template and primers 5′-ACGTT*CATATG*AAGTGGGATAGACTGTTTG-3′ and 5′-TC*GGATCC*CTACTTCTCCCTCTCCACAATATAG-3′. The newly introduced restriction sites for *Nde*I and *Bam*HI in the amplified gene (italicized in the primer sequences) were excised and then ligated into a pET-15b vector (Novagen, USA) to construct pET-15b-*gfps*. The recombinant expression of GFPP synthase in *E. coli* was performed using the same protocol as that for *cis*-prenyltransferase. The cells were harvested and disrupted by sonication in a HisTrap binding buffer. The enzyme was purified by heat-treatment and affinity chromatography with a HisTrap crude FF column in a manner similar to that for *cis*-prenyltransferase, with the exception of the lack of a second column-washing process. The level of purification was confirmed by 12% SDS–PAGE.

2.4. Preparation of radiolabeled GFPP

The reaction mixture containing 1.6 nmol of [1-^{14}C]IPP (2.04 GBq/mmol), 1.6 nmol of DMAPP, 1.5 μmol of $MgCl_2$, 30 μmol of MOPS-NaOH buffer, pH 7.0, and 320 ng of purified GFPP synthase, in a final volume of 300 μL, was incubated at 60 °C for 30 min. The reaction was stopped by chilling, and 400 μL of water saturated with NaCl was added. The mixture was extracted using 600 μl of 1-butanol saturated with NaCl-saturated water. [^{14}C]-GFPP in the butanol layer was used as a substrate for the prenyltransferase assay described below, after removal of butanol by complete evaporation. The amount of GFPP was calculated from the radioactivity of an aliquot of the butanol layer measured with a LSC-5100 liquid scintillation counter (Aloka, Japan).

2.5. Prenyltransferase assay

The standard assay mixture contained 0.1 nmol of [1-^{14}C]IPP (2.04 GBq/mmol), 0.01 nmol of (all-*E*) prenyl diphosphate (DMAPP, GPP, FPP, GGPP or [^{14}C]-GFPP), 1 μmol of $MgCl_2$, 20 μmol of MOPS-NaOH buffer, pH 7.0, 0.1% Triton X-100 and a suitable amount of purified *A. pernix cis*-prenyltransferase, in a final volume of 200 μl, was incubated at 60 °C for 30 min. The assay of GFPP synthase was performed in a similar manner with 0.4 nmol of each of the substrates, but in the absence of Triton X-100. The reaction was stopped by chilling, then 400 μL water saturated with NaCl was added. The mixture was extracted using 600 μl of 1-butanol saturated with NaCl-saturated water. The radioactivity in 10% of the butanol layer was measured using an LSC-5100 liquid scintillation counter (Aloka, Japan). The rest of the butanol layer was treated with potato acid phosphatase (Sigma–Aldrich, USA) according to the method of Fujii et al. [23], and the hydrolysates were then extracted with *n*-pentane to be analyzed by reversed-phase TLC using a precoated plate, LKC-18F or RP18, developed with acetone/H_2O (19:1). The distribution of radioactivity was detected using a Typhoon-FLA7000 multifunctional scanner (GE healthcare, USA).

2.6. Thermal stability analysis

The purified *cis*-prenyltransferase was treated with enterokinase (New England Biolabs, USA) in a buffer containing 20 mM Tris pH 7.2, 50% (v/v) glycerol, 200 mM NaCl, 2 mM $CaCl_2$ at 25 °C for 16 h, to remove the thioredoxin/polyhistidine/S-peptide tags fused at its N-terminus. The solution was heated at 55 °C for 1 h and then centrifuged to remove the enterokinase. The supernatant was concentrated using an Amicon Ultra-4 10K filter (Millipore, Ireland) and was then diluted with a HisTrap binding buffer repeatedly to exchange the buffer. The solution containing the excised protein was loaded on a HisTrap crude FF column to remove the tags. The flow-through fraction from the column was concentrated with an Amicon filter and treated at 65, 70, 75, 80, 90 or 100 °C for 1 h. The prenyltransferase assay of the heat-treated solutions was performed under the standard conditions described above, using FPP as the allylic primer substrate. The volume of each heat-treated

solution was set constant, but was controlled to insure that at least ~50% of both the substrates would remain unreacted.

3. Results and discussion

3.1. Substrate specificity of cis-prenyltransferase from A. pernix

The homologue of *cis*-prenyltransferase from *A. pernix*, which is encoded in the gene *ape_1385*, was recombinantly expressed in *E. coli*. Because *cis*-prenyltransferase is a membrane protein, detergent was added to the buffer for the disruption of *E. coli* cells to solubilize the protein. The protein was expressed in a fusion with thioredoxin/polyhistidine/S-peptide tags at the N-terminus. Thus it was purified by heat treatment and affinity chromatography toward the polyhistidine tag. Because the purity of the protein was not high, probably because of the coexistence of detergent, the standard protocol for affinity chromatography was modified by adding a second column-washing process with a buffer containing a higher concentration of imidazole. Nevertheless, protein bands with smaller molecular masses were still observed in the purified protein solution (Fig. 1A).

Prenyltransferase assay using the radiolabeled substrate [^{14}C]-IPP was performed with the purified *A. pernix cis*-prenyltransferase homologue, and showed that the protein catalyzed the condensation of [^{14}C]-IPP with the allylic primer substrate. Given the sequential homology, the archaeal protein is considered to be a *cis*-prenyltransferase. Thus, the substrate specificity of the enzyme, which was of our main interest, was analyzed. For the analysis, we cloned, recombinantly expressed, and purified GFPP synthase from *A. pernix* in advance (Fig. 1A), in order to synthesize [^{14}C]-GFPP. The enzymatically synthesized compound was used, together with other (all-*E*) prenyl diphosphates, to investigate the allylic substrate preference of *A. pernix cis*-prenyltransferase. The relative activity of the enzyme toward the various allylic substrates that are shown in Fig. 1B was calculated from the radioactivity extracted by butanol, using the number of [^{14}C]-IPP molecules that had been condensed with either the labeled or non-labeled allylic substrates. To determine the number of condensed IPP, the butanol-extracted products were analyzed by radio-TLC after phosphatase treatment (Fig. 1C, lane 1–5). The result from the radio-TLC analysis showed that the main product was UPP, regardless of the substrate. Thus, we referred to the enzyme as UPP synthase, but the *cis–trans* configurations of the double bonds of the product were not identical due to the differences in the allylic substrate, which were shown by the slightly different R_f values. The TLC analysis also confirmed that [^{14}C]-GFPP had been properly synthesized (Fig. 1C, lane 6). As shown in Fig. 1B, the most preferred allylic substrates were FPP and GFPP. DMAPP did not react with *A. pernix* UPP synthase, like known *cis*-prenyltransferases involved in glycosyl carrier lipid biosynthesis [19–22]. The reactivities of GPP and GGPP were significantly lower than those of FPP and GFPP. Then came the question of which compound was the physiological substrate for *A. pernix* UPP synthase. As shown by the radio-TLC analysis of the enzymatically synthesized [^{14}C]-GFPP after dephosphorylation (Fig. 1C, lane 6), the major product of *A. pernix* GFPP synthase was GFPP, with slight amounts of GGPP and C_{30} hexaprenyl diphosphate (HexPP) produced as by-products. The radioactive spot of FPP-derived alcohol was almost invisible. It should be emphasized that identical amounts of DMAPP and IPP were used for the synthesis of [^{14}C]-GFPP. Under reaction conditions such as this, shorter products tend to form by the shortage of IPP. This evidence suggests that the physiological substrate of *A. pernix* UPP synthase is likely GFPP rather than the shorter (all-*E*) prenyl diphosphate.

3.2. Characterization of A. pernix UPP synthase

The ratio of IPP to the allylic substrate was 10 in the above-described prenyltransferase assay to investigate the substrate preference of UPP synthase. However, it is known that the substrate ratio largely affects the product chain-length of longer-chain prenyl diphosphate synthases [21,24]. If the ratio of IPP to the allylic substrate becomes lower, the product will be shorter, probably because of the shortage of IPP or because of the frequent dissociation of an intermediate from the active site by competitive binding of the hydrophobic allylic substrate. Thus, we changed the substrate ratio and analyzed the product chain-length to understand the correlation between them (Fig. 2A). When the ratio of IPP to [^{14}C]-GFPP was decreased to 1, the UPP synthase predominately yielded shorter C_{30-45} products. When the ratio was increased to 100, the main product was C_{60} dodecaprenyl diphosphate. These results imply that the chain-length of the product of UPP synthase, which determines the structure of the glycosyl carrier lipid, is variable depending on the substrate ratio in the cells of *A. pernix*.

Because the enzyme had been heat-treated at 55 °C for 1 h through the purification process, the thermal stability of the enzyme from the hyperthermophile was examined by measuring

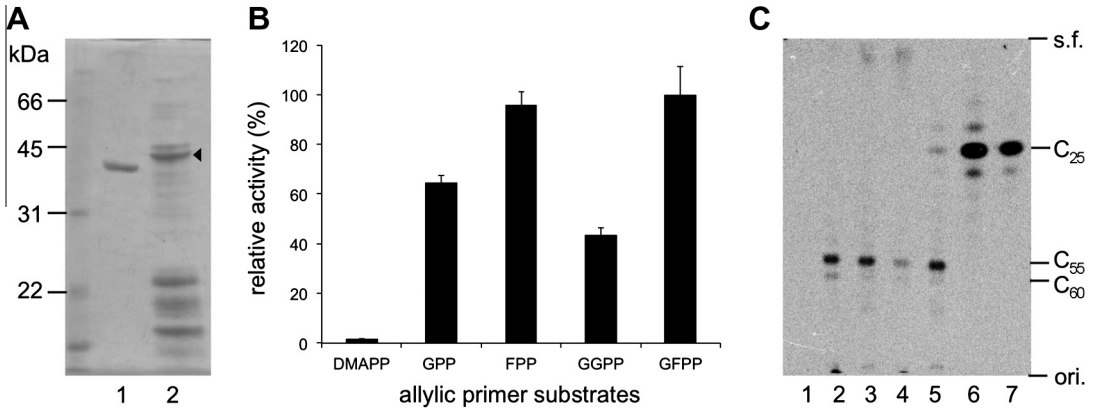

Fig. 1. Substrate specificity of *A. pernix cis*-prenyltransferase. (A) SDS–PAGE analysis of purified GFPP synthase (lane 1) and *cis*-prenyltransferase (lane 2). The arrowhead on lane 2 indicates the band of *cis*-prenyltransferase. (B) Relative activity of *cis*-prenyltransferase toward the allylic primer substrates. The activity toward GFPP (nmol/min/mg protein) was set at 100%. (C) Radio-TLC analysis of the products from the substrate preference test. The allylic primer substrate was as follows: lane 1, DMAPP; lane 2, GPP; lane 3, FPP; lane 4, GGPP; and, lane 5, [^{14}C]-GFPP. Lane 6 represents the dephosphorylation product from the enzymatically synthesized [^{14}C]-GFPP, which was used as a substrate for *cis*-prenyltransferase, and lane 7 represents authentic prenyl alcohols. Authentic geranylfarnesol (C_{25} alcohol) was prepared using GFPP synthase from *Methanosarcina mazei* following the reported procedure [27]. s.f., solvent front; ori., origin.

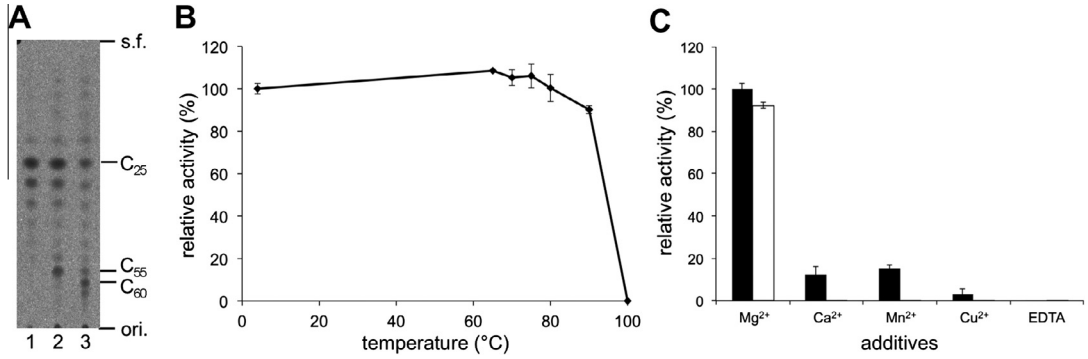

Fig. 2. Characterization of *A. pernix cis*-prenyltransferase. (A) Product chain-length was determined by TLC analysis. The ratio of IPP to GFPP was changed by using 0.01 nmol [^{14}C]-GFPP and various amounts of IPP as follows: lane 1, 0.01 nmol [^{14}C]-IPP; lane 2, 0.1 nmol [^{14}C]-IPP; and, lane 3, 1 nmol non-labeled IPP. The amount of the enzyme was controlled for each condition to avoid the depletion of the substrates. In each reaction, at least ~50% of both the substrates remained unreacted. s.f., solvent front; ori., origin. (B) Thermal stability was shown by the relative residual activity of the heat-treated enzyme. For the assay, FPP was used as the allylic substrate. The activity of the untreated (placed at 4 °C) enzyme was set at 100%. (C) The divalent metal ion requirement was investigated by replacing 5 mM $MgCl_2$ in the standard assay conditions by 5 (closed bars) or 50 mM (open bars) of various divalent metal ion (as chloride salt) or EDTA. FPP was used as the allylic substrate. The activity under the standard assay conditions was set at 100%.

the residual activity after additional treatment for 1 h at higher temperatures of 65, 70, 75, 80, 90 or 100 °C (Fig. 2B). Before the heat treatment, the affinity tags attached to the N-terminus of the enzyme were excised by protease treatment. The enzyme still retained >80% activity even after treatment at 90 °C. However, it was completely inactivated by treatment at 100 °C, which is in good agreement with the optimal growth temperature of *A. pernix* at 90–95 °C.

As shown in Fig. 2C, the requirements for a divalent metal ion were investigated. Complete inactivation caused by the addition of EDTA indicated that a divalent metal ion is necessary for the activity of *A. pernix* UPP synthase, as well as known *cis*-prenyltransferases. The addition of 5 mM Mg^{2+} gave the highest activity within the conditions tested. At the same concentration, Ca^{2+}, Mn^{2+} and Cu^{2+} all conferred significantly lower activity than Mg^{2+} does. A 10-fold higher concentration of Mg^{2+} continued to show activity that was comparable to 5 mM Mg^{2+}, while the other metal ions showed inhibitory effects at higher concentrations.

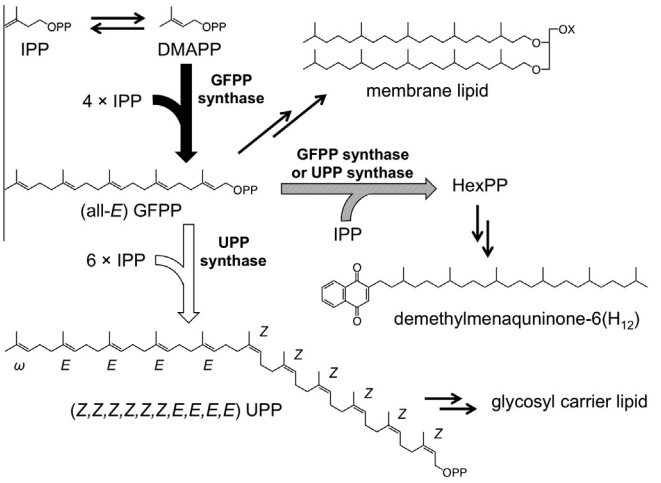

Fig. 3. Proposed biosynthetic pathway of isoprenoid compounds in *A. pernix*. The X and OPP in the chemical structures indicate the polar head groups of membrane lipids and a diphosphate group, respectively.

3.3. Speculation concerning the biosynthetic pathway of isoprenoid compounds in A. pernix

From the substrate specificity and product chain-length data of UPP synthase, the structure of the physiological product of the enzyme could be inferred, which determined the structure of the glycosyl carrier lipid of *A. pernix*. Given that the allylic primer substrate used in the cells of *A. pernix* was GFPP, which is one of the most favorable substrates *in vitro*, and that the chain-length of the physiological product was C_{55}, as it was in the *in vitro* study, the product should be (*Z,Z,Z,Z,Z,Z,E,E,E,E*)-UPP [(2*Z*,6*Z*,10*Z*,14*Z*,18*Z*,22*Z*,26*E*,30*E*,34*E*,38*E*)-3,7,11,15,19,23,27,31,35,39,43-undecamethyl-2,6,10,14,18,22,26,30,34,38,42-tetratetracontaundecaen-1-yl di phosphate] (Fig. 3). However, we could not exclude the possibility that the product might be longer or shorter than UPP, as could be deduced from the data in Fig. 2A, depending on the substrate ratio in the cells of *A. pernix*. Although the structure of the glycosyl carrier lipid from *A. pernix* remains unclear, our data strongly suggested that it would be a novel one. The chain-length and *cis–trans* configurations of the double bonds are of interest, as well as the specific reduction of a portion of the double bonds, which has been recently reported in association with glycosyl carrier lipids from other archaea [4–6].

When discussing the isoprenoid biosynthesis in *A. pernix*, it should be mentioned that the respiratory quinones of *A. pernix*, i.e., demethylmenaquinone and methionaquinone, have a fully or almost fully saturated C_{30} isoprenoid side-chain [25] (Fig. 3). Because the side-chain of respiratory quinones such as menaquinone and ubiquinone are known to be synthesized from (all-*E*) prenyl diphosphate, it seems reasonable to consider that GFPP synthase, which is the sole (all-*E*) prenyl diphosphate-producing enzyme in *A. pernix* and is involved in membrane lipid biosynthesis, is also responsible for the biosynthesis of C_{30} HexPP. Although the formation of the C_{30} product by *A. pernix* GFPS synthase has not been reported previously [17], our data showed that the enzyme can produce HexPP as a by-product, even when the ratio of IPP to DMAPP is 1 (Fig. 1C). Moreover, we also confirmed that a 10-fold increase in the substrate ratio results in a significant rise in the production of HexPP (data not shown). However, we could not exclude the possibility that the side-chain of the respiratory quinones of *A. pernix* might have arisen from (*Z,E*-mixed) HexPP. As shown in Fig. 2A, UPP synthase yielded (*Z,E*-mixed) HexPP as a by-product, particularly when the ratio of IPP to the allylic substrate was low. A reduction of almost all double bonds in the respiratory quinones from *A. pernix* did not allow us to obtain any information about the *cis–trans* double bond configuration of the precursor of the prenyl side-chain. Furthermore, a prenyltransferase

homologous to UbiA-family enzymes, most of which catalyze the transfer of a prenyl-chain to an aromatic quinone-head precursor, was recently found to use C_{50} (*Z,E*-mixed) decaprenyl monophosphate as a substrate [26]. Thus, it seems premature to draw any conclusions.

Acknowledgments

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