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A novel prenyltransferase, farnesylgeranyl diphosphate synthase, from the haloalkaliphilic archaeon, *Natronobacterium pharaonis*

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Abstract

A novel prenyltransferase, farnesylgeranyl diphosphate (FGPP) synthase (EC 2.5.1.X), which synthesizes C_{25}-prenyl diphosphate, was found in the haloalkaliphilic archaeon *Natronobacterium pharaonis*. It was separated from geranylgeranyl diphosphate (GGPP) synthase (EC 2.5.1.29), which synthesizes C_{20}-prenyl diphosphate, a major prenyltransferase in this organism. The highest activity of FGPP synthase was observed when GGPP was used as the allylic substrate. FGPP synthase may synthesize a precursor for the C_{25} moiety of C_{20},C_{25} diether lipids using a longer allylic diphosphate, such as GGPP synthesized by GGPP synthase, rather than dimethylallyl diphosphate, which is the product of isopentenyl diphosphate isomerase.

Key words: Prenyltransferase; Farnesylgeranyl diphosphate synthase; Geranylgeranyl diphosphate synthase; Archaeal lipid; Haloalkaliphile; *Natronobacterium pharaonis*

1. Introduction

Various types of prenyltransferases which catalyze C_5-unit condensation using isopentenyl diphosphate (IPP) and allylic diphosphate are known: geranyl diphosphate (GPP) synthase [1], farnesyl diphosphate (FPP) synthase [2], geranylgeranyl diphosphate (GGPP) synthase [3,4], and long-chain prenyltransferases [5–7] synthesizing prenyl diphosphates of C_{10}, C_{15}, C_{20}, and more than C_{25}, respectively. To our knowledge, however, there has been no report of prenyltransferase specifically synthesizing C_{25}-prenyl diphosphate (farnesylgeranyl diphosphate, FGPP), or FGPP synthase.

The haloalkaliphile *Natronobacterium pharaonis* [8] belongs to *Archaea,* which constitutes one of the three domains of living organisms [9], and has unique polar lipids, C_{20},C_{25} diether lipids [10], in addition to the C_{20},C_{20} diether lipids commonly occurring in *Archaea* [11] (Fig. 1). To date, little information is available concerning enzymes, including prenyltransferases, involved in the biosynthesis of the membrane lipids of haloalkaliphiles and other archaea. Earlier, we demonstrated for the first time that the methanogen *Methanobacterium thermoformicicum* SF-4 had only one prenyltransferase, GGPP synthase, which occupies the chain-elongation stage of isoprenoid biosynthesis for membrane polar lipids [3]. This prenyltransferase is responsible for the synthesis of the C_{20}-precursor for C_{20},C_{20} diether lipids [3,12]. In haloalkaliphiles, the C_{20} moiety of diether lipids may also be derived from GGPP synthesized by GGPP synthase. What synthesizes the C_{25} moiety has not yet been reported. In this article, we report the separation of specific FGPP synthase (EC 2.5.1.X) from GGPP synthase (EC 2.5.1.29) and its substrate and product specificities.

2. Experimental

2.1. Enzyme assay

[4-^{14}C]IPP was purchased from DuPont-New England Nuclear. Nonlabeled IPP, dimethylallyl diphosphate (DMAPP), GPP, FPP, and GGPP were synthesized by phosphorylation of the corresponding alcohols [3]. The enzyme activity was measured by determining the incorporation of [^{14}C]IPP into acid-labile allylic diphosphate. The assay mixture contained 75 mM Tris-HCl buffer (pH 7.6), 3 mM $MgCl_2$, 2.5 M KCl, 100 μM allylic diphosphate, 100 μM [^{14}C]IPP (66,000 dpm), and a suitable amount of enzyme in a final volume of 100 μl. After incubation at 37°C for 30 min, the reaction was stopped with 200 μl of conc. HCl/methanol mixture (1:4, v/v), and the samples were maintained at 60°C for 5 min to hydrolyze the products. The hydrolysates were extracted with 800 μl of ligroin, and a 640-μl portion of the extracts was counted for radioactivity with a scintillation counter.

2.2. Product analysis

For identification of the reaction products, a double-labeled experiment was carried out. [^{14}C]IPP (660,000 dpm) and [1-^{3}H]GGPP (American Radiolabeled Chemicals Inc., 660,000 dpm) were used as substrates. The assay mixture is described above. After the prenyltransferase reaction, the prenols obtained by alkaline phosphatase treatment

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Abbreviations: DMAPP, dimethylallyl diphosphate; FGPP, farnesylgeranyl diphosphate; FPP, farnesyl diphosphate; GGPP, geranylgeranyl diphosphate; GPP, geranyl diphosphate; IPP, isopentenyl diphosphate.

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of the products were separated by reversed-phase TLC (HPTLC RP-8) with a solvent system of acetone/water (95:5) [6]. The positions of authentic standards, geraniol, farnesol, geranylgeraniol (Kuraray), and ficaprenol (Sigma, mainly C_{50}–C_{60}), were visualized with iodine vapor. Iodine-positive spots and C_{25}–C_{45} positions on the TLC plate were scraped off and counted for radioactivity with a liquid scintillation counter.

2.3. Separation of FGPP synthase from GGPP synthase

N. pharaonis IFO 14720 (= DSM 2160, type strain) was grown in medium containing (per liter) 5 g of yeast extract, 5 g of casamino acids (Difco), 1 g of sodium glutamate, 2 g of KCl, 0.24 g of $MgSO_4 \cdot 7H_2O$, 0.17 g of $CaSO_4 \cdot 2H_2O$, 1 g of NH_4Cl, 5 g of Na_2CO_3, and 250 g of NaCl (pH 9.0) for 5 days at 37°C with shaking and harvested by centrifugation. Cell-free extracts were prepared by sonication in buffer A (50 mM Tris-HCl, pH 8.0, 2.5 M NaCl) containing 1.0 M $(NH_4)_2SO_4$. The solution was applied onto a column of Phenyl-Sepharose CL-4B (Pharmacia, 2.5 × 20 cm), and the column was thoroughly washed with buffer A containing 1.0 M $(NH_4)_2SO_4$. Active fractions were eluted with gradient 1.0–0 M $(NH_4)_2SO_4$ in buffer A. By this step, prenyltransferase activities were separated into two fractions as shown in Fig. 2. Each of the prenyltransferases was separately concentrated, applied onto a gel filtration column of Sephacryl S-300HR (Pharmacia, 2 × 105 cm) equilibrated with 50 mM Tris-HCl (pH 8.0) containing 2.5 M KCl, and eluted with the same buffer. Each of the enzymes was dialyzed against buffer B (50 mM potassium phosphate, pH 8.0, 2.5 M KCl) and applied onto a column of hydroxyapatite (Wako, 1.2 × 10 cm). The column was washed with buffer B, and elution was done with a gradient of phosphate concentration (50–200 mM).

3. Results

The separation of FGPP synthase, which could use GGPP as a substrate, from GGPP synthase, which does not use GGPP, was achieved by hydrophobic interaction chromatography (Fig. 2). The prenyltransferases were further purified by Sephacryl S-300 HR and hydroxyapatite chromatographies. FGPP synthase thus obtained was free of GGPP synthase, IPP isomerase, and phosphatases, and GGPP synthase was free of FGPP synthase, IPP isomerase, and phosphatases. FPP synthase was not found in this organism nor in methanogen [3]. Neither FGPP synthase nor GGPP synthase was a membrane-bound protein because both became weakly bound to the hydrophobic-interaction column. GGPP synthase activity was about 6-fold higher than FGPP synthase activity when 100 μM FPP and 100 μM IPP were used as substrates (Fig. 2).

By product analysis of FGPP synthase, the C_{25}-position on the TLC plate was double labeled by ^3H and ^{14}C at a nearly equal ratio (1:0.89) using [^{14}C]IPP with [^{3}H]GGPP as allylic partners (Fig. 3), indicating that double-labeled prenol is farnesylgeraniol. Radioactivities of more than C_{25}-positions were negligible. Stereochemistry of FGPP synthesized by FGPP synthase was unclear, but may be an all-*E* isomer because the same isomer was produced when DMAPP, GPP, and all-*E*-FPP, as well as all-*E*-GGPP, were used as substrates. Moreover, the stereochemistry of the C_{25} moiety of C_{20},C_{25}-diether lipids is identical with that of the C_{20} moiety.

The highest activity was obtained when GGPP and IPP were used as substrates, rather than shorter allylic diphosphate such as DMAPP (Table 1). The GGPP synthase used allylic diphosphates as substrates in the order of GPP > DMAPP > FPP; this order was the same as that of GGPP synthase of methanogen [3].

The molecular mass of FGPP synthase was estimated

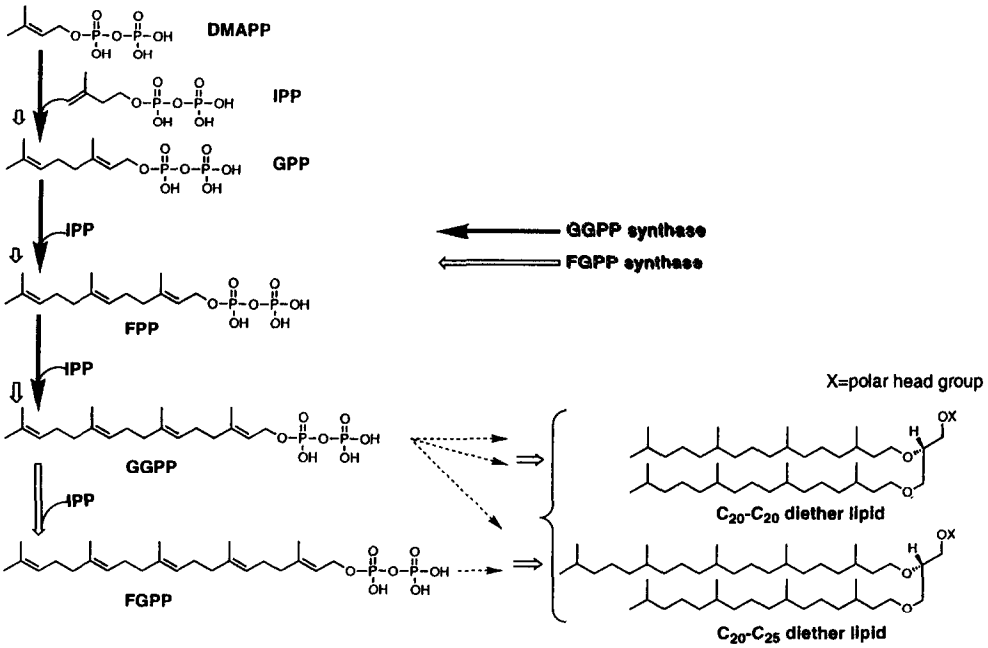

Fig. 1. Membrane polar lipid biosynthesis in *Natronobacterium pharaonis*. Isoprenoid chain elongation is catalyzed by two prenyltransferases, GGPP synthase and FGPP synthase. Former three steps, $C_5 \rightarrow C_{10} \rightarrow C_{15} \rightarrow C_{20}$, may be mainly responsible for GGPP synthase because of high activity of GGPP synthase than it of FGPP synthase. While, last step, $C_{20} \rightarrow C_{25}$, may be responsible for only FGPP synthase.

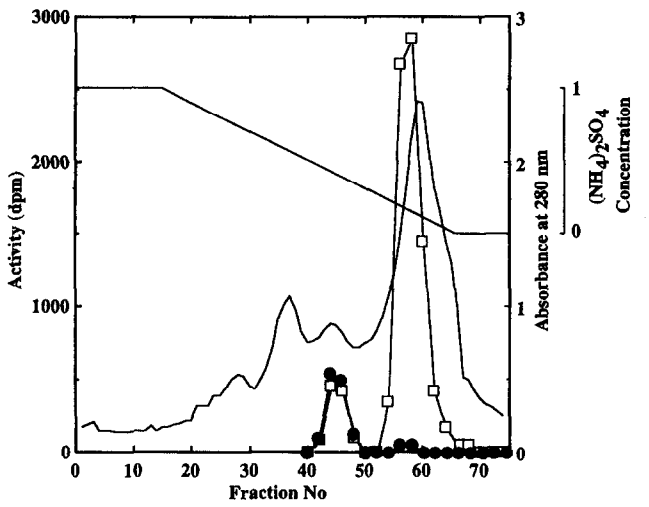

Fig. 2. Separation of FGPP synthase from GGPP synthase by Phenyl-Sepharose CL-4B column chromatography. ●, Assay using IPP and GGPP; □, assay using IPP and FPP; —, absorbance at 280 nm. Former prenyltransferase, FGPP synthase could use GGPP and FPP as allylic substrate, while, later one, GGPP synthase could use FPP, but little GGPP. Details are described in section 3.

to be 76 kDa by gel filtration on Sephacryl S-300HR (data not shown). The molecular mass of GGPP synthase was 68 kDa (data not shown).

4. Discussion

Novel prenyltransferase specifically synthesizing C_{25}-prenyl diphosphate, or FGPP synthase, was found for the first time in the haloalkaliphilic archaeon *N. pharaonis*. FGPP synthase was separated from GGPP synthase by hydrophobic interaction chromatography. As in the case of methanogen, FPP synthase was not found in this organism [3]. This feature may be a feature of *Archaea*. FGPP synthase and GGPP synthase may be involved in the production of precursors for membrane polar lipids, with the latter being the major prenyltransferase, as the polar lipids of this organism have C_{20} and C_{25} in the molar ratio of about 3:1 [13] (Fig. 1). The substrate specificity of haloalkaliphile GGPP synthase was similar to that of methanogen GGPP synthase, suggesting that both play similar roles in isoprenoid biosynthesis. However, the highest activity of the FGPP synthase was observed when GGPP was used as a substrate rather than the shorter allylic diphosphate. The FGPP synthase may specifically synthesized FGPP as the precursor for the C_{25} moiety of C_{20},C_{25} diether lipids using the longer allylic diphosphate such as GGPP synthesized with GGPP synthase rather than DMAPP, which is the product of IPP isomerase and a starting material for the isoprenoid chain elongation stage.

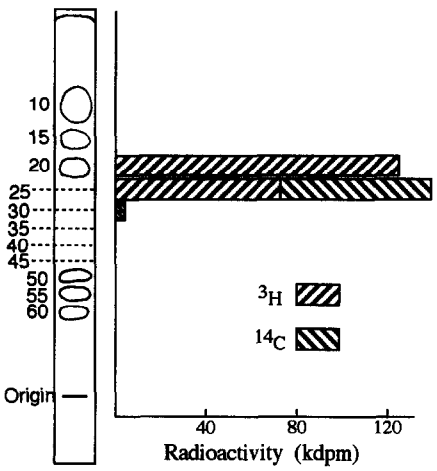

Fig. 3. Product analysis of FGPP synthase. [^{3}H]GGPP and [^{14}C]IPP were used as substrates. The C_{25}-position was double labeled by ^{3}H and ^{14}C. Geraniol, farnesol, geranylgeraniol, and ficaprenol (mainly C_{50}, C_{55}, and C_{60}) were used as authentic standards. Details are described in the text.

Table 1
Substrate specificities of FGPP synthase and GGPP synthase

Allylic Substrate	Concentration (μM)	Activity (dpm) FGPP synthase		GGPP synthase	
DMAPP	100	3,464	(55.3%)	6,294	(176,3%)
GPP	100	5,473	(87.4)	10,271	(287.8)
FPP	100	6,260	(100.0)	3,569	(100.0)
GGPP	100	6,763	(108.0)	31	(<1.0)
FPP+GGPP	100+100	8,762	(140.0)	2,307	(64.6)

Haloalkaliphilic archaea [10], including *N. pharaonis*, and non-alkaliphilic *Halococcus* [14] have C_{20},C_{25} diether lipids, in addition to C_{20},C_{20} derivatives commonly occurring in *Archaea* [11]. The chain-length specificity of the diether lipids may depend on the product specificity of prenyltransferases. Methanogens having only C_{20},C_{20} derivatives possess only one prenyltransferase, GGPP synthase [3], whereas *N. pharaonis* was found to have both FGPP synthase and GGPP synthase. Other haloalkaliphiles and *Halococcus* are also expected to possess both, while other archaea including methanogens not having C_{20},C_{25} derivatives may not possess FGPP synthase. In other words, the occurrence of FGPP synthase seems to be limited to haloalkaliphiles and *Halococcus*.

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