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Specificity of geranylgeranyl diphosphate synthase for homoallylic substrate analogs

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ABSTRACT

The goal of this study was to determine the substrate specificity of Homo sapiens geranylgeranyl diphosphate synthase (GGPPase) for analogs of isopentenyl diphosphate (IPP) to facilitate the application to organic synthesis techniques to the study of prenyl chain elongation enzymes. For this purpose, we used the IPP analogs 2a–d, which contain different alkyl side-chains at the 3-position, as substrates of the condensation reaction with the allylic substrate geranyl diphosphate (GPP) that is catalyzed by GGPPase. GGPPase catalyzed the reaction of GPP with 3-desmethylisopentenyl diphosphate (but-3-enyl diphosphate) to yield 3-desmethylfarnesyl diphosphate (12.1%), as well as the reaction of GPP with 3-ethylbut-3-enyl diphosphate or 3-propylbut-3-enyl diphosphate to yield 3-ethylfarnesyl diphosphate (46.9%) or 3-propylfarnesyl diphosphate (22.6%), respectively. However, a reaction product was not detected when 3-butylbut-3-enyl diphosphate was used as substrate.

1. Introduction

Approximately 40,000 isoprenoids (Scheme 1) are widely distributed among organisms. For example, prenyl diphosphate is a precursor in the biosynthetic pathways that produce steroids, carotenoids, dolichols, prenyl quinones, prenyl proteins, and natural rubber. Biosynthesis of these prenyl diphosphate polymers occurs through consecutive condensations of isopentenyl diphosphate (IPP) with its isomer dimethylallyl diphosphate (DMAPP) or with geranyl diphosphate (GPP). The stereospecific "head-to-tail" condensations proceed to form polymers of specific lengths, and prenyltransferases catalyze these reactions [1–6]. Because prenyltransferases exhibit substrate selectivity under mild reaction conditions, they will likely be useful for organic synthesis.

To exploit prenyltransferases for this purpose, our research has focused on the substrate specificity of farnesyl diphosphate synthase (FPPase) for artificial substrate analogs [7–12]. For example, we targeted geranylgeranyl diphosphate synthase (GGPPase) [EC 2.5.1.29], which catalyzes the early steps in biosynthetic pathways of the products described above as well as those of catabolic pathways, in concert with FPPase [13–15].

GGPPase catalyzes the sequential condensation of IPPs with the allylic substrates dimethylallyl diphosphate (DMAPP), geranyl diphosphate (GPP), or both to produce geranylgeranyl diphosphate (GGPP) [16,17]. An aspartate (D) residue-rich motif within *E*-type prenyl chain elongating enzymes (FPPase or GGPPase) may reside within the active sites of the enzymes [18–20]. The predicted primary structures of FPPase and GGPPase are similar [21–23], and they react with the same substrate. However, the detailed mechanism of the reaction catalyzed by GGPPase remains to be determined.

In order to review in which position of the active site of GGPPase the reaction product exists, we performed the docking simulations. Here, we examined the reactions catalyzed by GGPPase using 3-alkyl analogs of IPP as substrates and conducted enzyme-substrate docking simulations.

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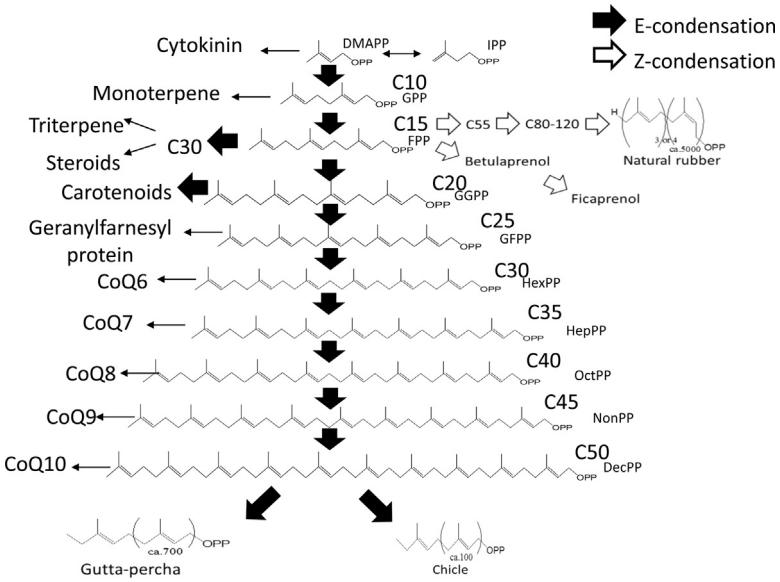

Scheme 1. Backbone subunits and bioactive isoprenoids.

2. Experimental

2.1. Analytical procedures

The prenyl alcohols produced by treating enzyme reaction products with alkaline phosphatase (Grade 1 from calf intestine, Roche) were measured using high-performance liquid chromatography (HPLC). Relative yields of the GGPPase reactions were calculated relative to those of GGPP produced by the standard enzymatic reaction of IPP with GPP. We used a LichroCART column (Merck-Japan, Tokyo, Japan) with a hexane:2-propanol (40:1) eluent for HPLC. Reaction products were identified using liquid chromatography-mass spectrometry (LC–MS) (Hitachi NanoFrontier LD, Hitachi High Technologies, Tokyo, Japan). All HPLC analyses were performed using a LaChrom Elite HPLC system (Hitachi L-7420, a Hitachi L-6000 pump and UV–vis spectroscopy detector), an Ulvac Chromato-DAQ II A/D converter (Ulvac Inc., Chigasaki, Japan), and a Merck LiChroCART column (Merck KGoA, Darmstadt, Germany).

GC–MS analysis was used to identify the prenyl alcohols derived from alkaline phosphatase treatment of the reaction products. The analytical system comprised a JEOL Q 1000GC-Md II Mass Spectrometer (Japan Electron Optics Laboratory Co. Ltd., Tokyo, Japan) with an HP-5 column (30 m × 0.32 mm$^{\varphi}$). A 1 ml/min helium carrier flow rate was used. The temperature gradient started with a 4.7-min hold at 50 °C, increased by 15 °C/min, and ended with a 2-min hold at 250 °C. Injector and GC-transfer-line temperatures were 200 °C.

2.2. Synthetic reactions

The 3-alkyl analogs of IPP, but-3-enyl- (**2b**); 3-ethylbut-3-enyl- (**2c**); 3-propylbut-3-enyl- (**2d**); and 3-butylbut-3-enyl diphosphates (**2e**), were synthesized according to published methods [7–10,24,25]. 3-DesmethylIPP was obtained by diphosphorylation using the method of Poulter et al. [26], after tosylating 3-buten-1-ol. 3-Ethyl-, 3-propyl-, and 3-butylbut-3-enyl diphosphates were synthesized according to a published method [9,10]. Briefly, 3-ethylbut-3-enyl diphsphate was synthesized as follows:2-(3-chloromethylbut-3-enoyloxy)-tetrahydro-2H-pyran was generated by selenium oxidation and chlorination of isopentenyl tetrahydropyranyl ether, which was methylated using a Grignard reaction to yield 3-ethylbut-3-en-1-ol after deprotection. The cognate alcohols were diphosphorylated [19].

2.3. Purification of GGPPases

Purification of human GGPPase was carried out according to a published method [23]. An expression plasmid obtained from Dr. H. Sagami of Tohoku University was used to transform *Escherichia coli* BL21 (DE3). Transformants were cultivated at 37 °C to an absorbance at 600 nm of 0.6. Isopropyl 1-thio-β-galactopyranoside was added to a final concentration of 1 mM, and then the cells were cultured at 37 °C overnight. The cultures were centrifuged, and the pelleted cells were suspended in lysis buffer (20 mM Tris–HCl, pH 7.9, 5 mM EDTA, and 1 mM phenylmethylsulfonyl fluoride) and were disrupted by sonication. His-tagged GGPPase was purified from the supernatant of the lysate using a His GraviTrap Column (GE Healthcare), and the fractions were subjected to SDS-PAGE. Coomassie Brilliant Blue staining revealed that the final preparation was 90% pure.

2.4. Enzyme assays

The reaction mixture for human GGPPase contained 125 μmol Tris–HCl buffer (pH 7.4), 50 μ mol β-mercaptoethanol, 25 μ mol $MgCl_2$, 1 μmol of each substrate analog (GPP **1** and IPP **2**, 3-alkyl analogs of IPP, **2a–e**), and GGPPase (approximately 128 μg) in a total volume of 1.0 ml. After incubation for 6 h at 37 °C, the reaction mixture was treated with alkaline phosphatase for 12 h, extracted with pentane, and analyzed using HPLC and GC–MS [8–10].

2.5. Enzyme-substrate docking simulations

The crystal structure of human GGPPase (PDB code: 2Q80PDB) was used for docking studies. Genetic Optimization of Ligand Docking (GOLD) [27] was employed to dock flexible ligands *in silico* to the binding sites of the enzymes to determine the possible binding mechanisms. Enzyme-substrate docking was visualized using PyMOL [28].