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Journal of Molecular Catalysis B: Enzymatic 19–20 (2002) 431–436

JOURNAL OF MOLECULAR CATALYSIS B: ENZYMATIC

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Dramatic changes in the substrate specificities of prenyltransferase by a single amino acid substitution

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Received 25 September 2001; received in revised form 23 May 2002; accepted 6 June 2002

Abstract

Farnesyl diphosphate (FPP) synthase catalyzes the condensation of isopentenyl diphosphate (IPP) with dimethylallyl diphosphate (DMAPP) or geranyl diphosphate (GPP) to give FPP as a final product. The FPP synthase of a thermophilic bacterium, *Bacillus stearothermophilus*, can hardly accept substrate analogs having oxygen atoms in their prenyl chain though the porcine FPP synthase can accept them.

We have prepared several point-mutated *B. stearothermophilus* FPP synthases, in which tyrosine was substituted with glycine (Y81G), serine (Y81S), arginine (Y81R) or aspartic acid (Y81D). Interestingly, the reactivities of the mutated FPP synthases were enhanced with respect to the substrate analogs having ω-oxygen atom in their prenyl chain (**1**–**4**).

Keywords: Farnesyl diphosphate synthase; Substrate specificity; Substrate analog of geranyl diphosphate; Mutant; Prenyltransferase

1. Introduction

Prenyltransferase catalyzes the condensation of an allylic diphosphate with isopentenyl diphosphate (IPP) stereospecifically, and the condensation terminates precisely until the elongation of prenyl chain reaches a certain length depending on the individual specificity of enzyme. These enzymes can be classified into two major types, (*E*)- and (*Z*)-prenyl chain elongation reactions [1,2]. Farnesyl diphosphate synthase (EC.2.5.1.10) is one of the short prenyl diphosphate synthases which is classified as a member of (*E*)-type prenyltransferases. It catalyzes the "head-to-tail" condensation of IPP with dimethylallyl diphosphate (DMAPP) or with geranyl diphosphate (GPP) to give all-(*E*) farnesyl diphosphate (FPP) as shown in Scheme 1 [1–3].

Porcine FPP synthase has been successfully applied to the stereospecific syntheses of several bioactive substances, such as faranal, the trail pheromone of Pharaoh's ant [4], the insect hormone, 4-methyl juvenile hormone I [5], and the butterfly hair pencil

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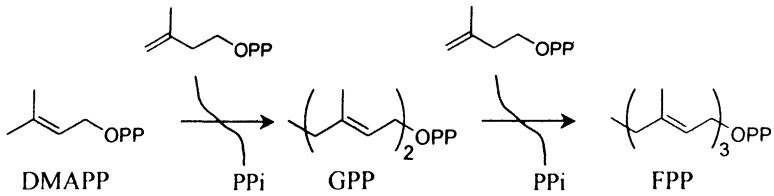
1381-1177/02/$ – see front matter
PII: S1381-1177(02)00195-9



Scheme 1. Farnesyl diphosphate synthase reaction.

pheromone [6]. Meanwhile, Koyama et al. [7] have carried out gene cloning, efficient overproduction in *Escherichia coli* cells and (its) purification of FPP synthase from a thermophilic bacterium *Bacillus stearothermophilus*. By comparison of the primary structures of many (*E*)-type prenyltransferases, they suggested that prenyltransferases have seven conservative motifs in their amino acid sequence [7], two of which are the most characteristic aspartate rich motifs. Then Ohnuma et al. [8] have reported that a tyrosine that is located at the fifth position upstream to the first aspartate rich motif, regulates the prenyl chain elongation by the hydrophobic interaction between the ω-terminal of the reaction product and the side chain of tyrosine. Amino acid substitution for this tyrosine caused the mutated FPP synthase to catalyze the synthesis of geranylgeranyl diphosphate or even longer prenyl diphosphates [8]. Although the thermostable FPP synthase from *B. stearothermophilus* has been shown to be useful as a synthetic catalyst [6,9] and applied to the one-pot synthesis of the sex pheromone like substance of a codling moth [10], these applications have been limited to the syntheses of such compounds having a hydrocarbon skeleton. On the basis of the findings reported by Ohnuma et al. [8], we anticipated that some mutated FPP synthases would change their substrate specificities and accept substrate analogs having a hydrophilic moiety in their alkyl chain if we replace the tyrosine at the position 81 (which corresponds to the fifth residue upstream to the first asprtate-rich domain) of the thermostable FPP synthase with other amino acid residues having a hydrophilic side chain. Thus, we have examined the four mutated *B. stearothermophilus*, Y81G, Y81R, Y81S, and Y81D, in which the tyrosine at position 81 are replaced with glycine, arginine, serine and aspartic acid, respectively. This paper describes the dramatic change of substrate specificities of the mutated FPP synthases.

2. Experimental

2.1. Chemicals

All-(*E*) GPP, all-(*E*) FPP and DMAPP were the same preparations as used in the previous works [8,10]. Substrate analogs of DMAPP or GPP, (*E*)-3,7-dimethyl-9-oxa-dodeca-2,6-dienyl diphosphate **1**, (*E*)-2,7-dimethyl-9-oxa-deca-2,6-dienyl diphosphate **2**, (*E*)-3,7-dimethyl-9.11-dioxa-dodeca-2,6-dimethyl-2,6 dienyl diphosphate **3**, (*E*)-8-hydroxy-3,7-dimethyl-octa-2,6-dienyl diphosphate **4** and (*E*)-3-methyl octa-3-enyl diphosphate **5** were synthesized according to the method reported previously [6,9,11]. Diphosphorylation of the corresponding alcohol was carried out by the method of Davisson et al. [13]. [1-^{14}C]IPP was purchased from Amersham Corp. Alkaline phosphatase was purchased from Sigma. Pre-coated reversed phase thin layer chromatography plates (LKC-18) were purchased from Whatman. Point-mutated enzymes, Y81G, Y81R, Y81S, and Y81D, were the same preparation to these prepared by the method reported previously [8].

2.2. Conditions of enzymatic reaction

The enzymatic activity was measured by determination of the amount of [1-^{14}C]IPP incorporated into hexane-extractable hydrolysates derived from the enzymatic product after acid hydrolysis. The incubation mixture for the FPP synthase reaction contained in a final volume of 1.0 ml, 50 mM of Tris–HCl buffer (pH 8.5), 50 μmol of $MgCl_2$, 50 μmol of 2-mercaptoethanol, 25 nmol of GPP or a substrate analog to be examined, 25 nmol of [1-^{14}C]IPP (specific activity 37 GBq/mol) and a suitable amount of the wild-type or a mutated FPP synthase. After incubation at 55 °C for 15 min, the reaction was terminated

by addition of HCl to the mixture. Then the reaction mixture was treated with hexane, and the radioactivity of the hexane extract was measured by liquid scintillation counter.

2.3. Purification of FPP synthase and the mutants, Y81G, Y81R, Y81S, and Y81D

E. coli JM 105 cells haboring the wild-type or the mutated *B. stearothermophilus* gene, which had been incubated and treated with isopropyl-β-D-thiogalactosylpyranoside to overproduce the prenyltransferase, were disrupted by sonication. Then the cell-free extract was collected by centrifugation (10,000 × *g*), treated with heat, and fractionated with 35–75% ammonium sulfate, followed by two chromatographies (Butyl- and DEAE-Toyopearl). The protein fraction was analyzed for purity by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) with Coomassie Brilliant Blue staining [7]. The mutated synthases, Y81G, Y81R, Y81S, and Y81D, show similar chromatographic properties to those of the wild-type FPP synthase during purification.

2.4. Product analysis

After the enzymatic reaction at 55 °C for 2 h, radioactive prenyl diphosphate was hydrolyzed to the corresponding alcohols with alkaline phosphate according to the method described in the previous paper [12]. The hydrolysate was extracted with pentane and analyzed by reversed phase thin layer chromatography with LKC-18 pre-coated TLC (Whatman) in a solvent system of acetone/H_2O (4/1). The positions of the authentic standard alcohols were visualized with iodine-vapor, and distribution of radioactivity was detected with a bio-image analyzer BAS 1000Mac (Fuji Film).

3. Results and discussion

The four mutated enzymes studied in this work showed comparable levels of prenyltransferase activities, when the reaction was carried out using GPP as an allylic substrate, which were similar to those reported by Ohnuma et al. [8].

We synthesized five analogs of GPP or DMAPP (**1**–**5**) according to the method in the previous reports [6,9,11,17], whose structures are as shown in Fig. 1, and studied the substrate specificities of the wild-type and the mutated FPP synthase from *B. stearothermophilus* using these substrate analogs. Relative activities as an allylic substrate for the wild-type and the mutated synthases are summarized in Table 1.

Table 1 shows the relative reactivities of the wild-type and the mutated FPP synthases for the analogs compared to the enzymatic activities when GPP was employed as the allylic substrate. As shown in Table 1, the wild-type FPP synthase scarcely accepted the analog (**1**–**4**) as primer substrate compared to the natural substrate GPP. On the other hand, the mutated FPP synthases, Y81G, Y81R, Y81S, and Y81 accepted the analog **2** as a preferable substrate to give the FPP analog. Especially, Y81G, Y81S, and Y81D showed increased activities for the analog **2** with 30-, 17-, 31-folds higher rates than the wild-type enzyme, respectively. The condensation of the analog **3** with IPP was also catalyzed more effectively by Y81R, Y81S, and Y81D, with 12-, 15.5- and 15-folds increased activities as compared with the wild-type enzyme. Y81R effectively catalyzed the condensation of the analog **4** with IPP with 4.9-fold increased

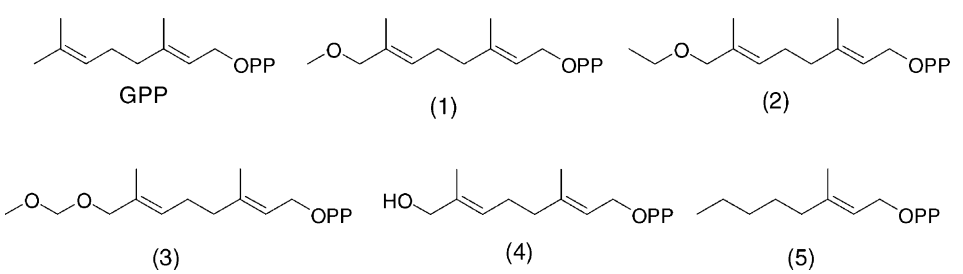


Fig. 1. GPP and substrate analogs studied in this work.

Table 1
Relative activities of wild-type and mutated FPP synthases (Y81G, Y81R, Y81S and Y81D) with GPP or the analogs (**2**–**5**) as allylic substrate and the product distribution patterns in each reaction

Analog	Relative activity[a]	F-type	GG-type	GF-type	H-type
GPP					
W.T-FPS	100.0	100.0	ND	ND	ND
Y81G-FPS	32.7	52.7	30.8	16.5	ND
Y81R-FPS	55.9	34.7	65.3	ND	ND
Y81S-FPS	28.9	25.4	67.4	7.2	ND
Y81D-FPS	68.4	76.0	24.0	ND	ND
2 (1)[b]					
W.T-FPS	3.0	100.0	ND	ND	ND
Y81G-FPS	90.6	5.1[b]	90.3[b]	4.7[b]	ND
Y81R-FPS	39.0	97.3	2.7	ND	ND
Y81S-FPS	50.6	36.6	63.4	ND	ND
Y81D-FPS	93.7	100.0	ND	ND	ND
3					
W.T-FPS	2.5	100.0	ND	ND	ND
Y81R-FPS	30.8	99.3	0.7	ND	ND
Y81S-FPS	38.7	59.3	40.7	ND	ND
Y81D-FPS	37.5	100.0	ND	ND	ND
4					
W.T-FPS	19.7	100.0	ND	ND	ND
Y81R-FPS	96.7	51.4	48.6	ND	ND
Y81S-FPS	8.1	8.4	75.9	8.0	7.7
Y81D-FPS	12.6	86.3	13.7	ND	ND
5					
W.T-FPS	111.0	99.2	0.8	ND	ND
Y81G-FPS	24.1	18.5	40.1	41.4	ND
Y81R-FPS	5.2	18.2	81.8	ND	ND
Y81S-FPS	3.4	16.0	63.5	20.5	ND
Y81D-FPS	6.0	12.1	87.9	ND	ND

F-type indicates the product incorporated with one molecule of IPP. GG-type indicates the product of one isoprene-unit longer than F-type compound. GF-type indicates one isoprene unit longer than GG-type compound, three unit prenyl chain elongation product. H-type has one isoprene unit longer than GF-type compound. ND: not detected.

[a] Relative activities of mutated FPP synthase with GPP or substrate are shown when the activity of the wild-type enzyme is shown 100.

[b] Analog **1** was used for Y81G.

activity, whereas, Y81S and Y81R were moderately active with **4**. On the other hand, as shown in Table 1, the wild-type enzyme catalyzed the condensation of the analog **5** with IPP as effectively as that of GPP as reported in the previous reports [6,9]. Although Y81G showed moderate activity to **5**, the other mutants, Y81R, Y81S, and Y81D, accepted the analog **5** with reduced activity.

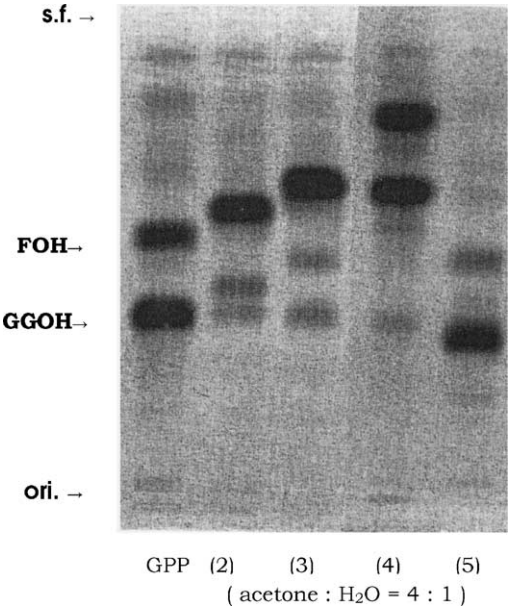


Fig. 2. TLC-autoradiochromatograms of alcohols by enzymatic hydrolysis of reaction products catalyzed by Y81R-FPP synthase. Analyzed by reversed phase TLC LKC-18 as described in "Section 2".

For product analysis, the alcohols obtained by enzymatic hydrolysis of the diphosphate products, formed by the reaction with the mutated FPP synthases, were analyzed by TLC-autoradiography. The autoradiogram of the alcohols derived from the products catalyzed by Y81R is as shown in Fig. 2. Most of all the mutants having a replacement at the position 81 of the FPP synthase from *B. stearothermophilus* have been reported to give longer chain product than intrinsic product. The condensation of GPP with IPP catalyzed by Y81R gave two major products, FPP (C_{15}) and GGPP (C_{20}) as shown by lane 1 in Fig. 2. On the other hand, when analog **2** was employed, the major product was 12-ethoxyfarnesyl diphosphate, which is the condensation product having a one prenyl unit chain elongation (F-type), with a detectable amount of 16-ethoxygeranylgeranyl diphosphate (GG-type; as shown by lane 2 in Fig. 2). These results are essentially similar to those reported previously by using the pig liver enzyme [6,9,11]. Similarly, with the analogs **3** or **4**, the enzymatic products corresponding to F- and GG-types were detected at a ratio of 97:3

and 99:1, respectively (as shown by lanes 3 and 4 in Fig. 2). Analysis of the products obtained from the reaction catalyzed by mutants Y81G, Y81S, and Y81D gave essentially similar results to those of Y81R (data not shown). The ratio of the product distribution of the condensations catalyzed by the mutants is listed in Table 1. Y81D catalyzed production of the F-type product using **2** or **3** as the allylic substrates. However, Y81S in the condensation between IPP and **2** or **3**, gave a mixture of F-type and GG-type products in approximately equal yield. On the other hand, Y81G was found to be tolerant enough to the analog **4** having a hydroxyl group at the ω-end of GPP, to produce even longer chain products, GF-type as well as H-type, which are the condensation product with 3- and 4-prenyl units chain elongation, respectively.

The crystal structure of avian FPP synthase has been determined to 2.6 Å resolution by Tarshis et al. [14]. The FPP synthase is composed of 13 α-helices joined by connecting loops, and eight of them form a large cavity. Two aspartate-rich motifs that are highly conserved among the prenyltransferase family are formed on opposite walls of this cavity and the active site of FPP synthase seems to be located in the large central cavity. Ohnuma et al. [15] showed a considerable structural similarity between avian and *B. stearothermophilus* FPP synthase and indicated that the tyrosine of FPP synthase from *B. stearothermophilus* is situated at a location 11–12 Å apart from the first aspartate-rich motif, which has been proposed to bind the diphosphate moiety of substrate via magnesium ion complex formation [16]. Furthermore, Ohnuma et al. [15] has indicated that the amino acid at position 81 of the wild-type FPP synthase from *B. stearothermophilus* contacts directly with the ω-terminus of an elongated product by hydrophobic interaction. On the basis of their report, we can postulate that a hydrophilic side chain of the amino acid at position 81 (arginine, serine, and aspartic acid) contacts favorably with an ω-oxygen atom in the prenyl chain elongating product, not blocking a chain elongation for the hydrophobic prenyl moiety (Fig. 3B). On the other hand, the wild-type enzyme easily accepts the analog **5** having a hydrophobic moiety in its prenyl chain, but the mutants (Y81R, Y81S, and Y81D) can hardly accept the analog **5** as a primer substrate. A possible model for the interaction between the hydrophobic ω-end of the chain elongating product and hydrophobic side chain at position 81 is as

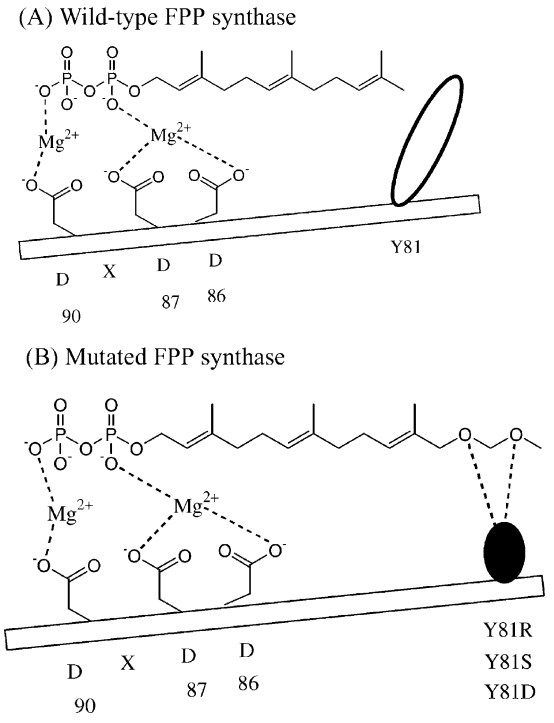


Fig. 3. Relationship of substrate analogs and mutated FPP synthases: (A) wild-type FPP synthases; (B) mutated FPP synthases.

shown in Fig. 3A. On the other hand, Y81G also accepts the analogs having a hydrophilic moiety to afford a longer-chain product than those of other mutants. In this case, the mutant enzyme lacks the block for the chain elongation as suggested by Ohnuma et al. [8,15].

4. Conclusion

In this study, we have demonstrated that a single amino acid substitution dramatically altered the substrate specificity of the FPP synthase from *B. stearothermophilus*. Although the wild-type FPP synthase can hardly accept substrates having a hydrophilic moiety in their prenyl chain (**1**, **2**, **3**, and **4**), the mutants, Y81G, Y81R, Y81S, and Y81D can easily accept those. These results suggest that the mutated FPP synthases would be more applicable to the organic synthesis of bioactive substances having an ω-oxygen atom in their prenyl chain such as the butterfly hair pencil pheromone, as compared to the wild-type enzyme or the pig liver enzyme [6].

Acknowledgements

This work was supported in part by a Grand-in-Aid for Scientific Research (12680587 to Yuji Maki). We are grateful to Ms. Keiko Satoh, Ms. Shoko Kikuchi, and Mr. Hirosi Sakazume for the preparation of enzymes and syntheses of the substrate analogs.

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