A Role of the Amino Acid Residue Located on the Fifth Position before the First Aspartate-rich Motif of Farnesyl Diphosphate Synthase on Determination of the Final Product*

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Farnesyl diphosphate (FPP) synthase catalyzes consecutive condensations of isopentenyl diphosphate with allylic substrates to give FPP, C-15 compound, as a final product and does not catalyze a condensation beyond FPP. Recently, it was observed that, in Bacillus stearothermophilus FPP synthase, a replacement of tyrosine with histidine at position 81, which is located on the fifth amino acid before the first aspartate-rich motif, caused the mutated FPP synthase to catalyze geranylgeranyl diphosphate (C-20) synthesis (Ohnuma, S.-i., Nakazawa, T., Hemmi, H., Hallberg, A.-M., Koyama, T., Ogura, K., and Nishino, T. (1996) J. Biol. Chem. 271, 10087–10095). Thus, we constructed 20 FPP synthases, each of which has a different amino acid at position 81, and analyzed them. All enzymes except for Y81P can catalyze the condensations of isopentenyl diphosphate. The final products and the product distributions are different from each other. Y81A, Y81G, and Y81S can produce hexaprenyl diphosphate (C-30) as their final product. The final product of Y81C, Y81H, Y81I, Y81L, Y81N, Y81T, and Y81V are geranylgeranyl diphosphate (C-25), and Y81D, Y81E, Y81F, Y81K, Y81M, Y81Q, and Y81R cannot produce polyprenyl diphosphates more than geranylgeranyl diphosphate. Substitution of tryptophan does not affect the product specificity of FPP synthase. The average chain length of products is inversely proportional to the accessible surface area of substituted amino acid. However, no significant relation between the final chain length and the kinetic constants $K_m$ and $V_{max}$ are observed. These observations strongly indicate that the amino acid does not come into contact with the substrates but directly contacts the $\omega$-terminal of an elongating allylic product. This interaction must prevent further condensation of isopentenyl diphosphate.

Isoprenoids, which include over 23,000 known metabolites, are the most chemically diverse family of naturally occurring compounds. The essential and major biosynthetic step in all isoprenoid metabolism is the elongation of isoprene units by prenyltransferases (Fig. 1) (1). These enzymes, which consecutively or mediate alkylation of isopentenyl diphosphate (IPP, C-5) by allylic diphosphates, are classified according to the chain length of the final product and the stereochemistry of double bond formed by the condensations. So far, a number of prenyltransferases have been determined from various organisms.

For example, farnesyl diphosphate (FPP) synthase (EC 2.5.1.1) catalyzes the sequential condensations of two molecules of IPP (C-5) with dimethylallyl diphosphate (DMAPP, C-5) to give a C-15 compound with E-stereochemistry. The product, FPP, occupies a central point leading to several branches of the pathway for the synthesis of important classes of compounds, including sterols, farnesylated proteins, hemes, respiratory quinones, sesquiterpenes, and dolichols. On the other hand, geranylgeranyl diphosphate (GGPP, C-20) synthase (EC 2.5.1.29) catalyzes the condensation of IPP to give (all-\(E\))-GGPP, which plays as a precursor for carotenoids, chlorophylls, geranylgeranylated proteins, and archaeobacterial membrane lipids.

These prenyltransferases catalyze the same sort of condensation and have a similarity in amino acid sequences (2, 3). However, every enzyme does not catalyze a further condensation of IPP than the general ultimate product. Until now it has been left in question how the consecutive condensations precisely stop at a destined step.

Recently, our group succeeded in converting FPP synthase from Bacillus stearothermophilus to GGPP synthase using chemical random mutagenesis followed by an in vivo color selection (4). From the analysis of the mutations in the FPP synthases whose product specificities had become the same as GGPP synthase, we defined three amino acids that could determine the final chain length; leucine at position 34, tyrosine at position 81, and valine at position 157. In particular, the mutated enzyme that has a substitution of histidine for tyrosine at position 81, which is situated at the fifth amino acid before the first aspartate rich consensus motif, the most effectively produces GGPP. Moreover, our group also showed that, in the case of Sulfolobus acidocaldarius GGPP synthase, the amino acid at the same position also determines the chain length of the product, GGPP (5). Thus, in this paper, we precisely analyze the role of the amino acid at position 81 of B. stearothermophilus FPP synthase on chain length determination.

EXPERIMENTAL PROCEDURES

Materials—Precoated reversed phase thin-layer chromatography plates, LKC-18 were purchased from Whatman (all-\(E\))-FPP, (all-\(E\))-DMAPP, (all-\(E\))-GGPP, (all-\(E\))-FPP synthase.

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1 The abbreviations used are: IPP, isopentenyl diphosphate; DMAPP, dimethylallyl diphosphate; GPP, geranyl diphosphate; FPP, farnesyl diphosphate; GGPP, geranylgeranyl diphosphate; GFPP, geranylferneyl diphosphate; HPP, hexaprenyl diphosphate.
GGPP, GPP, and DMAPP were the same preparations as used in the previous works (6, 7). [1-14C]IPP was purchased from Amersham Corp. pTV118N was purchased from Takara Shuzo Co., Ltd. All other chemicals were of analytical grade.

**Construction of the Mutated FPP Synthase by Site-directed Mutagenesis**—Site-directed mutagenesis was performed according to Kunkel's method (8). Single strand DNA coding the wild-type FPP synthase gene was generated by the M13KO7 helper phage infection of CJ236 cells that contained pFPS (4). The resulting antisense single strand DNA template was isolated and purified by standard methods. The synthetic sense oligonucleotides designed to produce the desired point mutations were as follows:

- **Y81A**: 5'-GATCCATACG-GCTTCTTGTGATCATGATTTTG-3'
- **Y81C**: 5'-GATCCATACG-TGCCTCTTGTGATCATGATTTTG-3'
- **Y81D**: 5'-GATCCATACG-GACCTCTTGTGATCATGATTTTG-3'
- **Y81E**: 5'-GATCCATACG-GAACCTCTTGTGATCATGATTTTG-3'
- **Y81F**: 5'-GATCCATACG-TACTCTTGTGATCATGATTTTG-3'
- **Y81G**: 5'-GATCCATACG-GGTCTCTTGTGATCATGATTTTG-3'
- **Y81H**: 5'-GATCCATACG-GTTCTCTTGTGATCATGATTTTG-3'
- **Y81I**: 5'-GATCCATACG-TATCTCTTGTGATCATGATTTTG-3'
- **Y81K**: 5'-GATCCATACG-AATCTCTTGTGATCATGATTTTG-3'
- **Y81L**: 5'-GATCCATACG-CTCTTGTGATCATGATTTTG-3'
- **Y81M**: 5'-GATCCATACG-ATCTCTTGTGATCATGATTTTG-3'
- **Y81N**: 5'-GATCCATACG-GATCTCTTGTGATCATGATTTTG-3'
- **Y81P**: ND
- **Y81Q**: 5'-GATCCATACG-CACTCTTGTGATCATGATTTTG-3'
- **Y81R**: 5'-GATCCATACG-GAGCTCTTGTGATCATGATTTTG-3'
- **Y81S**: 5'-GATCCATACG-GTCCTCTTGTGATCATGATTTTG-3'
- **Y81T**: 5'-GATCCATACG-ACCTCTTGTGATCATGATTTTG-3'
- **Y81V**: 5'-GATCCATACG-GTCTCTTGTGATCATGATTTTG-3'
- **Y81W**: 5'-GATCCATACG-GTCTCTTGTGATCATGATTTTG-3'

These substituted codons are frequently used in *Escherichia coli*. Mutated positions are indicated in bold face. New recognition sites of BspHI are introduced in all primers and are underlined. After mutagenesis, all mutants were confirmed by digestion with BspHI and DNA sequencing. A mutated enzyme with Y81H was previously reported (4).

**Preparation and Purification of Mutated FPP Synthase—** *E. coli* DH5a was transformed with the plasmids carrying mutated FPP synthase genes and cultured according to the methods described previously (9). The cells were harvested without isopropyl-1-thio-β-D-galactopiranose induction and disrupted by sonication in 50 mM Tris-HCl.

**Table I**

Prenyltransferase activity of the mutated FPP synthase

| Enzyme | Specific activity | Relative activity |
|--------|------------------|------------------|
|        | nmol/min/mg | DMAPP | GPP | FPP |
| Wild type | 10.70 | 149.00 | 100 | 3.55 |
| Y81A | 17.50 | 84.00 | 100 | 78.60 |
| Y81C | 14.60 | 181.00 | 100 | 102.00 |
| Y81D | 9.15 | 166.00 | 100 | 60.20 |
| Y81E | 11.20 | 98.00 | 100 | 87.30 |
| Y81F | 4.86 | 145.00 | 100 | 56.10 |
| Y81G | 7.84 | 181.00 | 100 | 102.00 |
| Y81H | 8.15 | 166.00 | 100 | 60.20 |
| Y81I | 10.70 | 145.00 | 100 | 56.10 |
| Y81K | 14.60 | 181.00 | 100 | 102.00 |
| Y81L | 4.86 | 145.00 | 100 | 56.10 |
| Y81M | 11.20 | 98.00 | 100 | 87.30 |
| Y81N | 14.60 | 181.00 | 100 | 102.00 |
| Y81P | 8.15 | 166.00 | 100 | 60.20 |
| Y81Q | 11.20 | 98.00 | 100 | 87.30 |
| Y81R | 4.86 | 145.00 | 100 | 56.10 |
| Y81S | 7.84 | 181.00 | 100 | 102.00 |
| Y81T | 8.15 | 166.00 | 100 | 60.20 |
| Y81V | 11.20 | 98.00 | 100 | 87.30 |
| Y81W | 14.60 | 181.00 | 100 | 102.00 |

ND, not detected.
buffer, pH 7.0, containing 10 mM 2-mercaptoethanol and 1 mM EDTA. The homogenate was heated at 55°C for 60 min and then centrifuged at 100,000 × g for 10 min. The supernatant was used as a heat treated enzyme to determine prenyltransferase activity. For further purifications of the mutated enzymes (Y81G, Y81A, Y81S, and Y81F), E. coli cells treated with isopropyl-1-thio-β-D-galactopyranoside were used. The purification scheme is essentially the same as that described previously (see Table II) (3). Each of the mutated FPP synthases was confirmed to be homogeneous by SDS-polyacrylamide gel electrophoresis (12.5%) with Coomassie Brilliant Blue staining. These mutated FPP synthases, Y81G, Y81A, Y81S, and Y81F, showed similar chromatographic properties to those of wild-type enzyme during the purification procedures.

**Determination of Prenyltransferase Activity**—The enzyme activity was measured by determination of the amount of [1-14C]IPP incorporated into 1-butanol-extractable polypropenyl diphosphates according to the method described previously (3). One unit of enzyme activity was defined as the activity required to incorporate 1 nmol of [1-14C]IPP into 1-butanol-extractable products when the assay was carried out using GPP as an allylic substrate.

**Product Analysis**—After enzymatic reaction at 55°C, the polyprenyl diphosphates were extracted with 1-butanol, and then the 1-butanol was evaporated under a N2 stream. The resulting polyprenyl diphosphates were treated with acid phosphatase according to the method of Fujii et al. (10). The hydrolysates were extracted with pentane and analyzed by reversed phase thin layer chromatography.

**Fig. 2.** TLC-aroraiochromatograms of the alcohols obtained by enzymatic hydrolysis of the products formed by reaction of the mutated FPP synthase. The sample from incubation of 25 μM [1-14C]IPP (37 GBq/mol) and 25 μM DMAPP (A), GPP (B), or FPP (C) with the indicated mutated enzyme was analyzed by reversed phase LKC-18 TLC as described under "Experimental Procedures." Spots of authentic standard alcohols: GOH, geraniol; FOH, farnesol; GGOH, geranylgeraniol; GFOH, geranyl-farnesol; HOH, hexaprenol. Ori., origin; S.F., solvent front.
Product Specificity of Mutated FPP Synthases

Using LKC-18 developed with acetone/H₂O (9/1). Authentic standard alcohols were visualized with iodine vapor, and the distribution of radioactivity was detected with a Bio-image analyzer BAS2000 (FUJIFILM).

RESULTS

Construction of 20 Enzymes That Have Different Amino Acids at Position 81—Wild-type FPP synthase of B. stearothermophilus catalyzes the condensation of IPP to give FPP (C-15) as the longest and the major product. During the investigation of the mechanism of final chain length determination, we found that a replacement of tyrosine with histidine at position 81, which is situated at the fifth amino acid before the first aspartate rich motif, brought about a change of product specificity. The mutated enzyme can effectively catalyze the condensation of IPP with FPP to give GGPP (C-20). Therefore, in order to understand the role of the amino acid at position 81, nineteen recombinants were constructed by means of site directed mutagenesis using synthetic DNA primers, each of which contains a mutated codon and a new recognition site for BspHI. After mutageneses, the sequences were confirmed by dideoxy method.

Determination of Chain Length of Products Obtained from Reaction with Mutated FPP Synthases—Cells of E. coli DH5α were transformed with the plasmids encoding the mutated FPP synthase and cultured. Crude enzyme solutions were prepared through sonicating the cultured cells. Since the B. stearothermophilus FPP synthase is thermostable, the cell homogenate was heated at 55 °C for 60 min prior to the enzyme assay to inactivate thermolabile prenyltransferases derived from the host cells. After heat treatment, the amounts of mutated FPP synthases were determined by SDS-polyacrylamide gel electrophoresis (12.5%, data not shown). The supernatant fractions contained almost all of the FPP synthase, indicating that all mutant FPP synthases are thermostable. Moreover, expression levels of the mutated FPP synthases are similar. The specific activities of the heat-treated enzymes, which were determined by measuring the radioactivity in 1-butanol extractable materials of the reaction using DMAPP, GPP, or FPP as a primer substrate, are shown in Table I. When the assay was carried out using GPP, all enzymes except for Y81P showed comparable levels of prenyltransferase activities. When assayed using FPP, considerable activities were observed in almost all mutated FPP synthases. These results indicate that the mutated enzymes can catalyze the condensation beyond FPP. On the other hand, relative activities for DMAPP are different. The activities of wild-type enzyme, Y81D, Y81E, Y81F, and Y81K for DMAPP are higher than those for GPP, whereas the activities of Y81I and Y81R are lower. Y81P did not show any prenyltransferase activity. Since it has been reported that the region around the first aspartate rich domain forms an α-helix (11), this loss of activity seems to result from destruction of the secondary structure by the substitution of proline.

After the digestion of 1-butanol extractable products by the action of acid phosphatase, the chain length distribution of the resultant alcohols and their amounts were analyzed by reversed phase TLC. Prenyltransferases are supposed to utilize even initially formed intermediates as primer substrates. To avoid this interference, prenyltransferase reaction was terminated within a time when 10% of substrates were consumed. As shown in Fig. 2, a variety of final products and product distri-

![Graph showing relationship between average chain length and accessible surface area](image)

**Table II**

| Total protein | Total activity | Specific activity | Purification | Recovery |
|--------------|---------------|------------------|--------------|----------|
| mg           | μmol/min      | nmol/min/mg      | -fold        | %        |
| **Y81G**     |               |                  |              |          |
| Crude lysate | 1080.00       | 436.0            | 368          | 1.00     | 100.00 |
| 100,000 × g supernatent | 742.00 | 290.0 | 403 | 1.23 | 68.60 |
| Butyl-Toyopearl | 212.00 | 95.6 | 451 | 1.23 | 21.90 |
| Mono-Q       | 19.20         | 43.0             | 2340         | 6.36     | 9.86  |
| **Y81A**     |               |                  |              |          |
| Crude lysate | 2200.00       | 637.0            | 290          | 1.00     | 100.00 |
| 100,000 × g supernatent | 1470.00 | 487.0 | 331 | 1.14 | 76.50 |
| Butyl-Toyopearl | 78.80 | 67.2 | 855 | 2.95 | 8.35  |
| Mono-Q       | 57.40         | 53.2             | 927          | 3.20     | 8.35  |
| **Y81F**     |               |                  |              |          |
| Crude lysate | 1410.00       | 451.0            | 320          | 1.00     | 100.00 |
| 100,000 × g supernatent | 718.00 | 269.0 | 375 | 1.17 | 59.60 |
| Butyl-Toyopearl | 91.10 | 134.0 | 1470 | 4.59 | 29.70 |
| Mono-Q       | 14.10         | 30.9             | 2190         | 6.84     | 6.85  |
| **Y81W**     |               |                  |              |          |
| Crude lysate | 1020.00       | 220.0            | 216          | 1.00     | 100.00 |
| 100,000 × g supernatent | 499.00 | 128.0 | 257 | 1.19 | 58.20 |
| Butyl-Toyopearl | 23.20 | 27.3 | 1180 | 5.46 | 12.40 |
| Mono-Q       | 5.10          | 12.0             | 2350         | 10.90    | 5.45  |
Product Specificity of Mutated FPP Synthases

Table III: Kinetic Constants of the Mutated FPP Synthase

The concentration of IPP employed as a homoallylic substrate.

The concentration of allylic substrate.

The concentration of IPP used in the experiments using GPP or FPP as an allylic substrate.

The concentration of IPP used in the experiments using DMAPP as an allylic substrate.

The concentrations of IPP, DMAPP, GPP, FPP, and GGPP employed in the experiments using DMAPP as an allylic substrate.

The concentrations of IPP, DMAPP, GPP, FPP, and GGPP employed in the experiments using FPP as an allylic substrate.

The concentrations of IPP, DMAPP, GPP, FPP, and GGPP employed in the experiments using GGPP as an allylic substrate.

The concentration of IPP employed as a homoallylic substrate.

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the purification procedures.

Kinetic constants of the purified mutants for IPP and allylic substrates (DMAPP, GPP, FPP, and GGPP) were determined by measuring the radioactivities in the 1-butanol-extractable products (Table III). In these experiments, the counter-substrate was used at high concentration enough for $K_m$ of the counter-substrate. The concentrations of counter-substrates are indicated in Table III. As expected, Y81A and Y81G could accept GGPP as an allylic substrate. The Michaelis constants for GGPP are lower than those for other substrates. However, both enzymes showed the highest $V_{\max}$ values when GPP was used, and the activity for GGPP was the lowest among all allylic substrates. In Y81W, although the mutation did not alter the final product, kinetic constants were changed. Especially, the apparent $K_m$ value for DMAPP was dramatically increased. In Y81F, the $V_{\max}$ value for FPP is half of that for GPP, and the apparent $K_m$ value for FPP is similar to that for GPP.

Although there were several interesting observations from kinetic analysis of these mutated FPP synthases, no relationship between the chain length of the final product and the apparent Michaelis constant was observed.

**DISCUSSION**

In this study, we have demonstrated that the chain length of a final product is dramatically changed by replacement of only one amino acid at position 81, which is situated at the fifth amino acid before the first aspartate-rich motif, and have indicated that the average chain length of products is inversely proportional to the accessible surface area of the substituted amino acid.

The crystal structure of avian FPP synthase has been determined to 2.6-A resolution by Tarshis et al. (11). The FPP synthase is composed of 13 $\alpha$-helices jointed by connecting loops, and eight of them form a large central cavity. Two aspartate-rich motifs that are highly conserved among the prenyltransferase family are found on opposite walls of this cavity, and the active site of FPP synthase seems to be located in the large central cavity. In a previous report (4), our group showed a considerable structural similarity between avian and B. stearothermophilus FPP synthases and indicated that the tyrosine-81 of B. stearothermophilus FPP synthase is situated on a point 11–12 Å apart from the first aspartate-rich motif, which has been thought to bind the diphosphate moiety of an allylic substrate via the magnesium ion (1, 16–18). The distance is similar to the length of the hydrocarbon moiety of FPP. From these data, we suggested that the aromatic ring of tyrosine-81 might prevent more chain elongations than FPP. It supports this hypothesis that an amino acid having smaller accessible surface area can form a longer final product. Moreover, the fact that a mutated GGPP synthase with an aliphatic amino acid at that position yields products slightly longer than that with a nonaliphatic amino acid with a similar accessible surface area indicates that the amino acid at position 81 directly contacts with the $\omega$-terminal of elongating product by hydrophobic interaction. On the basis of this, we propose a simple model for the condensation and termination mechanism of the FPP synthase reaction (Fig. 4).

The deduced amino acid sequence analysis of cloned prenyltransferase genes shows that all prenyltransferases have a significant sequence similarity. Three prenyltransferases that catalyze consecutive condensations beyond GGPP have been cloned, hexaprenyl diphosphate synthase from Saccharomyces cerevisiae (19), heptaprenyl diphosphate synthase from B. stearothermophilus (20), and octaprenyl diphosphate synthase from E. coli (21). These enzymes have alanine at the position corresponding to position 81 of B. stearothermophilus FPP synthase. These data strongly suggest that this amino acid primarily enables the enzymes to catalyze the formation of products longer than GGPP, and that other amino acid sequences of these enzymes should determine the final product to be C-30,

**FIG. 4. Simple model for consecutive condensations of IPP by FPP synthase.** First, FPP synthase accepts IPP and DMAPP. After two consecutive condensations of IPP, the $\omega$-terminal of the formed FPP reaches the amino acid at the fifth amino acid before the first aspartate rich motif. If the side chain of the amino acid is large, further condensation is inhibited, resulting in formation of FPP (route A). On the other hand, when the side chain is small, the condensation continues to give long products such as HPP (route B).
C-35, or C-40.

We have mainly been discussing the final products of mutated FPP synthases. However, product distribution was also affected by mutation. For example, Y81M produces primarily GGPP from DMAPP. On the other hand, Y81N yields three products, FPP, GGPP, and GFPP. Thus, we checked whether or not the divergence of products of each mutated enzyme is related to other factors. The divergence values were not related to the accessible surface area, the hydrophobicity (14), conformational preference parameters for secondary structure (15), or specific activity for DMAPP, GPP, and FPP. It has been reported regarding some prenyltransferases that the amounts of intermediate products are affected by reaction conditions, including substrate concentration, although the destined final product is not altered. Presumably, the mutations cause additional structural changes that affect the affinity of the enzyme for reaction intermediates. Further analysis should be done to understand the reason.

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