

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 geranylfarnesyl 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 ω -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

mediate alkylation of isopentenyl diphosphate (IPP, C-5)¹ 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*)-

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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, geranylfarnesyl diphosphate; HPP, hexaprenyl diphosphate.

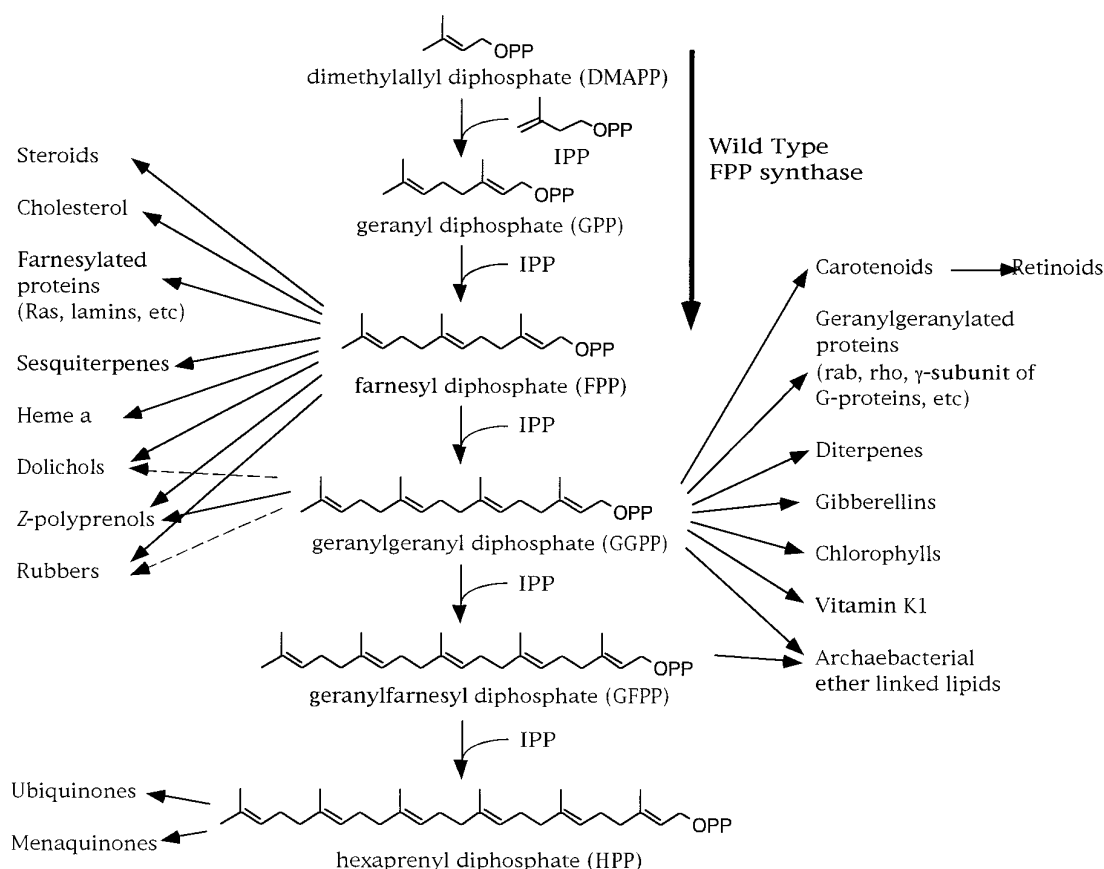


FIG. 1. Consecutive condensation of isopentenyl diphosphates by prenyltransferase. Wild-type FPP synthase of *B. stearrowthermophilus* catalyzes consecutive condensations of IPP to produce FPP as the ultimate product.

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 M13K07 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'-GATCCATACGGCTTCTTTGATTCATGATGATTTG-3'; Y81C, 5'-GATCCATACGCTGCTCTTTGATTCATGATGATTTG-3'; Y81D, 5'-GATCCATACGACTCTTTGATTCATGATGATTTG-3'; Y81E, 5'-GATCCATACGGAATCTTTGATTCATGATGATTTG-3'; Y81F, 5'-GATCCATACGTTCTCTTTGATTCATGATGATTTG-3'; Y81G, 5'-GATCCATACGGGTTCTTTGATTCATGATGATTTG-3'; Y81I, 5'-GATCCATACGATTTCTTTGATTCATGATGATTTG-3'; Y81K, 5'-GATCCATACGAAGTCTTTGATTCATGATGATTTG-3'; Y81L, 5'-GATCCATACGCTCTCTTTGATTCATGATGATTTG-3'; Y81M, 5'-GATCCATACGATGCTCTTTGATTCATGATGATTTG-3'; Y81N, 5'-GATCCATACGAACTCTTTGATTCATGATGATTTG-3'; Y81P, 5'-GATCCATACGCGTCTTTGATTCATGATGATTTG-3'; Y81Q, 5'-GATCCATACGCAATCTTTGATTCATGATGATTTG-3'; Y81R, 5'-GATCCATACGAGGTCTTTGATTCATGATGATTTG-3'; Y81S, 5'-GATCCATACGTCTCTTTGATTCATGATGATTTG-3'; Y81T, 5'-GATCCATACGACATCTTTGATTCATGATGATTTG-3'; Y81V, 5'-GATCCATACGGTGTCTTTGATTCATGATGATTTG-3'; Y81W, 5'-GATCCATACGTGGTCTTTGATTCATGATGATTTG-3'. These substituted codons are frequently used in *Escherichia coli*. Mutated positions are indicated in bold face. New recognition sites of *Bsp*HI are introduced in all primers and are underlined. After mutagenesis, all mutants were confirmed by digestion with *Bsp*HI and DNA sequencing. A mutated enzyme with Y81H was previously reported (4).

Preparation and Purification of Mutated FPP Synthase—*E. coli* DH5α 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-galactopyranoside induction and disrupted by sonication in 50 mM Tris-HCl

TABLE I
Prenyltransferase activity of the mutated FPP synthase

Prenyltransferase activities of the 20 FPP synthases were measured using heat-treated enzymes as described under "Experimental Procedures." These activities were determined from the amount of radioactivity incorporated into 1-butanol-extractable materials when 25 μM of [1-¹⁴C]IPP and 25 μM of the indicated allylic substrate were used. The left column indicates the specific activity obtained from the reaction using GPP as an allylic substrate. The right three columns indicate the relative activity of DMAPP, GPP, or FPP, which is based on GPP activity.

Enzyme	Specific activity nmol/min/mg	Relative activity		
		DMAPP	GPP	FPP
Wild type	10.70	149.00	100	3.55
Y81A	17.50	84.00	100	78.60
Y81C	14.60	107.00	100	59.30
Y81D	7.84	181.00	100	102.00
Y81E	9.15	166.00	100	60.20
Y81F	11.20	145.00	100	56.10
Y81G	17.90	98.00	100	87.30
Y81H	2.31	47.70	100	102.00
Y81I	4.39	8.14	100	132.00
Y81K	4.86	193.00	100	114.00
Y81L	10.70	34.90	100	63.80
Y81M	14.50	114.00	100	64.80
Y81N	8.17	118.00	100	75.10
Y81P	ND ^a	ND	ND	ND
Y81Q	12.00	121.00	100	63.50
Y81R	4.18	21.30	100	113.00
Y81S	15.30	105.00	100	88.40
Y81T	14.40	107.00	100	46.60
Y81V	9.65	77.50	100	77.20
Y81W	11.70	118.00	100	3.13

^a ND, not detected.

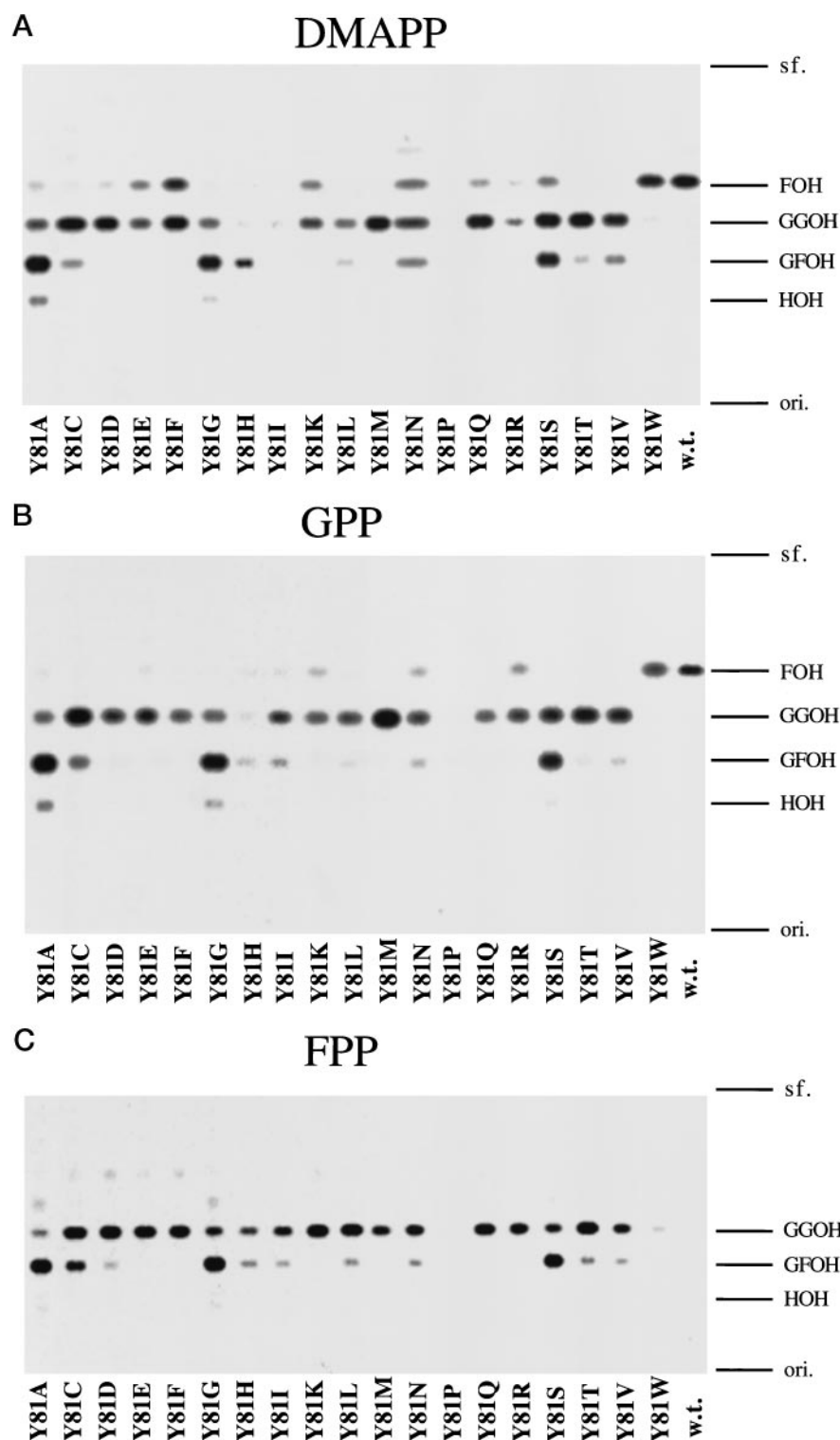


FIG. 2. TLC-autoradiochromatograms of the alcohols obtained by enzymatic hydrolysis of the products formed by reaction of the mutated FPP synthase. The sample from incubation of 25 μ M [14 C]IPP (37 GBq/mol) and 25 μ M DMAPP (A), GPP (B), or FPP (C) with the indicated mutated enzyme was analyzed by reversed 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.

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 $\times g$ 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 [14 C]IPP incorporated into 1-butanol-extractable polyprenyl diphosphates according to the method described previously (3). One unit of enzyme activity was defined as the activity required to incorporate 1 nmol of [14 C]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 N_2 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

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

*Bsp*HI. 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* DH5 α 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-

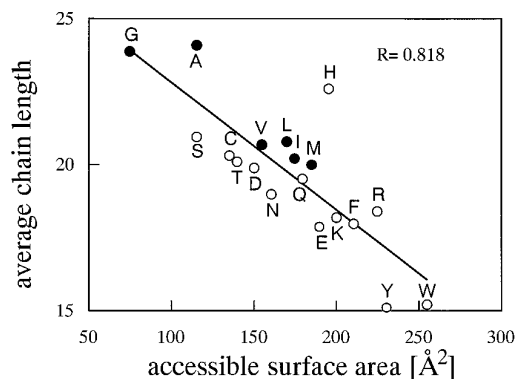


FIG. 3. Relationship between average chain length of products and accessible surface area. Shown is the relationship between an accessible surface area and an average chain length of products that are obtained from DMAPP. Values of accessible surface areas reported by Chothia (13) are used. Values of average chain length are determined by the equation described under "Results." The regression line was made from 19 determinations, except for Y81P, by the least squares method. Aliphatic and nonaliphatic amino acids are indicated as closed and open circles, respectively.

TABLE II
Purification of mutated B. stearothermophilus FPP synthases

Four mutated FPP synthases, Y81G, Y81A, Y81F, Y81W, were purified as described under "Experimental Procedures." Prenyltransferase activity was determined from the amount of radioactivity incorporated into 1-butanol extractable materials when GPP was used as an allylic substrate.

	Total protein	Total activity	Specific activity	Purification	Recovery
	mg	$\mu\text{mol/min}$	nmol/min/mg	-fold	%
Y81G					
Crude lysate	1080.00	436.0	368	1.00	100.00
100,000 $\times$ g supernatant	742.00	299.0	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 $\times$ g supernatant	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 $\times$ g supernatant	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 $\times$ g supernatant	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

TABLE III
 Kinetic constants of the mutated FPP synthase

Kinetic constants of the five purified mutated FPP synthases were determined as described under "Experimental Procedures." $V_{\max}$ was determined from the amount of radioactivity incorporated into 1-butanol extractable materials when [1- 14 C]IPP and the indicated allylic substrate were used. Each value represents the mean $\pm$ S.D. of three determinations.

Enzyme	DMAPP			GPP			FPP			GGPP		
	K_m	$V_{\max}$	IPP ^a	K_m	$V_{\max}$	IPP ^a	K_m	$V_{\max}$	IPP ^a	K_m	$V_{\max}$	IPP ^a
	μM	$nmol\ min^{-1}\ mg^{-1}$	μM	μM	$nmol\ min^{-1}\ mg^{-1}$	μM	μM	$nmol\ min^{-1}\ mg^{-1}$	μM	μM	$nmol\ min^{-1}\ mg^{-1}$	μM
Wild type ^b	12.9 $\pm$ 1.20	7400 $\pm$ 1810	100	10.4 $\pm$ 1.50	8990 $\pm$ 528	50	ND ^c	ND		ND	ND	
Mutant 1 ^b	341 $\pm$ 75.6	2170 $\pm$ 511	500	66.3 $\pm$ 4.80	4200 $\pm$ 748	100	17.3 $\pm$ 3.20	1620 $\pm$ 100	25	ND	ND	
Mutant 4 ^b	179 $\pm$ 39.4	1830 $\pm$ 118	500	10.1 $\pm$ 0.10	1610 $\pm$ 143	100	5.6 $\pm$ 0.30	891 $\pm$ 7.0	25	ND	ND	
Y81W	689 $\pm$ 56.7	1300 $\pm$ 67.7	1000	4.60 $\pm$ 0.55	1080 $\pm$ 55.6	240	ND ^c	ND		ND	ND	
Y81A	16.8 $\pm$ 3.50	2110 $\pm$ 397	100	5.88 $\pm$ 1.4	2290 $\pm$ 441	200	2.44 $\pm$ 0.37	1180 $\pm$ 91.7	180	2.07 $\pm$ 0.036	42.5 $\pm$ 1.18	150
Y81G	9.79 $\pm$ 0.30	1260 $\pm$ 51.4	100	12.2 $\pm$ 2.70	3750 $\pm$ 184	220	4.06 $\pm$ 0.32	1320 $\pm$ 46.0	200	0.217 $\pm$ 0.013	31.7 $\pm$ 1.24	300
Y81F	11.5 $\pm$ 3.00	965 $\pm$ 184	120	4.61 $\pm$ 0.75	1260 $\pm$ 85.3	220	4.48 $\pm$ 0.26	720 $\pm$ 39.4	180	ND	ND	

Enzyme	IPP ^d			IPP ^e			IPP ^f			IPP ^g		
	K_m	$V_{\max}$	DMAPP ^h	K_m	$V_{\max}$	GPP ^h	K_m	$V_{\max}$	FPP ^h	K_m	$V_{\max}$	GGPP ^h
	μM	$nmol\ min^{-1}\ mg^{-1}$	μM	μM	$nmol\ min^{-1}\ mg^{-1}$	μM	μM	$nmol\ min^{-1}\ mg^{-1}$	μM	μM	$nmol\ min^{-1}\ mg^{-1}$	μM
Wild type ^b	11.7 $\pm$ 1.90	7460 $\pm$ 1470	500	10.6 $\pm$ 0.50	10400 $\pm$ 115	100	ND	ND		ND	ND	
Mutant 1 ^b	265 $\pm$ 3.50	4790 $\pm$ 7.0	450	12.4 $\pm$ 0.80	3150 $\pm$ 54.0	250	9.40 $\pm$ 1.10	687 $\pm$ 39.0	35	ND	ND	
Mutant 4 ^b	27.9 $\pm$ 2.10	3800 $\pm$ 70.0	450	15.3 $\pm$ 1.30	2860 $\pm$ 81	100	6.1 $\pm$ 1.00	783 $\pm$ 76.0	25	ND	ND	
Y81W	31.8 $\pm$ 3.20	1060 $\pm$ 71.3	1600	3.96 $\pm$ 0.44	2130 $\pm$ 164	140	ND	ND		ND	ND	
Y81A	4.79 $\pm$ 0.30	2040 $\pm$ 21.5	100	4.19 $\pm$ 0.45	2710 $\pm$ 294	120	2.14 $\pm$ 0.50	1060 $\pm$ 76.5	60	29.2 $\pm$ 2.17	128 $\pm$ 16.2	60
Y81G	9.47 $\pm$ 1.40	2870 $\pm$ 258	140	4.50 $\pm$ 0.34	3210 $\pm$ 119	80	1.66 $\pm$ 0.25	937 $\pm$ 75.4	40	133 $\pm$ 14.5	464 $\pm$ 39.1	60
Y81F	5.48 $\pm$ 0.78	1380 $\pm$ 311	140	2.20 $\pm$ 0.14	1710 $\pm$ 30.9	140	1.11 $\pm$ 0.20	730 $\pm$ 41.8	60	ND	ND	

^a The concentration of IPP employed as a homoallylic substrate.

^b Previously reported (4).

^c ND, not determined.

^d For the reaction with DMAPP.

^e For the reaction with GPP.

^f For the reaction with FPP.

^g For the reaction with FPP.

^h The concentration of allylic substrate.

butions were observed. When DMAPP was used as a primer, Y81A, Y81G, and Y81S produced hexaprenyl diphosphate (HPP, C-30) as the longest product. The ratios of HPP among the all products were small, and the main product was geranylarnesyl diphosphate (GFPP, C-25). Y81A and Y81G formed greater amounts of HPP and GFPP than did Y81S. The final product of Y81C, Y81H, Y81I, Y81L, Y81N, Y81T, and Y81V was GFPP, but the product distributions were different. Y81H produced GFPP as the major product, whereas the major product of the other six mutated GGPP synthases was GGPP. Also, the amount of FPP formed by Y81N was much larger than those of the other six mutants. Y81D, Y81E, Y81F, Y81K, Y81M, Y81Q, and Y81R produced GGPP as the final and major product. Substitution of tryptophan did not introduce any significant change in product specificity. Similar observations were made in the experiments using GPP or FPP as an allylic substrate (Fig. 2, B or C). Moreover, as described below, we purified four mutated FPP synthases (Y81W, Y81A, Y81G, and Y81F) homogeneously. The products formed by these purified enzymes were almost similar to those from partial purified enzymes in any case (data not shown).

Relationship between Average Chain Length and Accessible Surface Area—From the results of Fig. 2, we felt that mutated GGPP synthases with small amino acids at position 81 seemed to form a longer final product than those with bulky amino acids. Thus, the relationship between the average chain length of products obtained from every mutated enzyme and the accessible surface area of the amino acid residue at the mutated position was investigated. Accessible surface area of an atom is defined as the area over which the center of a water molecule can be placed while retaining van der Waals contacts with that atom and not penetrating any other atom (12). The areas of 20 amino acids were reported by Chothia (13) and are known to be simply proportional to the two-thirds power of their molecular

weights. The average chain length of products of each mutated enzyme is determined by the equation described below.

$$\begin{aligned} \text{Average chain length} = & \text{ratio of GPP} \times 10 + \\ & \text{ratio of FPP} \times 15 + \\ & \text{ratio of GGPP} \times 20 + \\ & \text{ratio of GFPP} \times 25 + \\ & \text{ratio of HPP} \times 30 \end{aligned}$$

As shown in Fig. 3, when DMAPP was used as a primer substrate, the average chain length was inversely proportional to accessible surface area of the substituted amino acid. The coefficient of correlation, obtained from 19 determinations except for Y81P, is 0.818. Similar observations were obtained from the results using GPP (coefficient of correlation, 0.887) or FPP as an allylic substrate (data not shown). In Fig. 3, aliphatic amino acids (alanine, valine, leucine, isoleucine, methionine) are indicated by *closed circles*. Almost all these amino acids are situated over the regression line, indicating that the enzymes having aliphatic amino acids at position 81 formed the product longer than other enzymes. We also investigated the relation to hydrophobicity of the amino acids (14), conformational preference parameters for secondary structure (15), and specific activity for DMAPP, GPP, and FPP. No relationship between the average chain length and these values was observed.

Kinetic Constants of Mutated FPP Synthases—In order to characterize the mutants in detail, four representative mutated FPP synthases, Y81A, Y81G, Y81W, and Y81F, were purified homogeneously (Table II). All the mutated FPP synthase, Y81G, Y81A, Y81W, and Y81F, showed similar chromatographic properties to those of the wild-type enzyme during

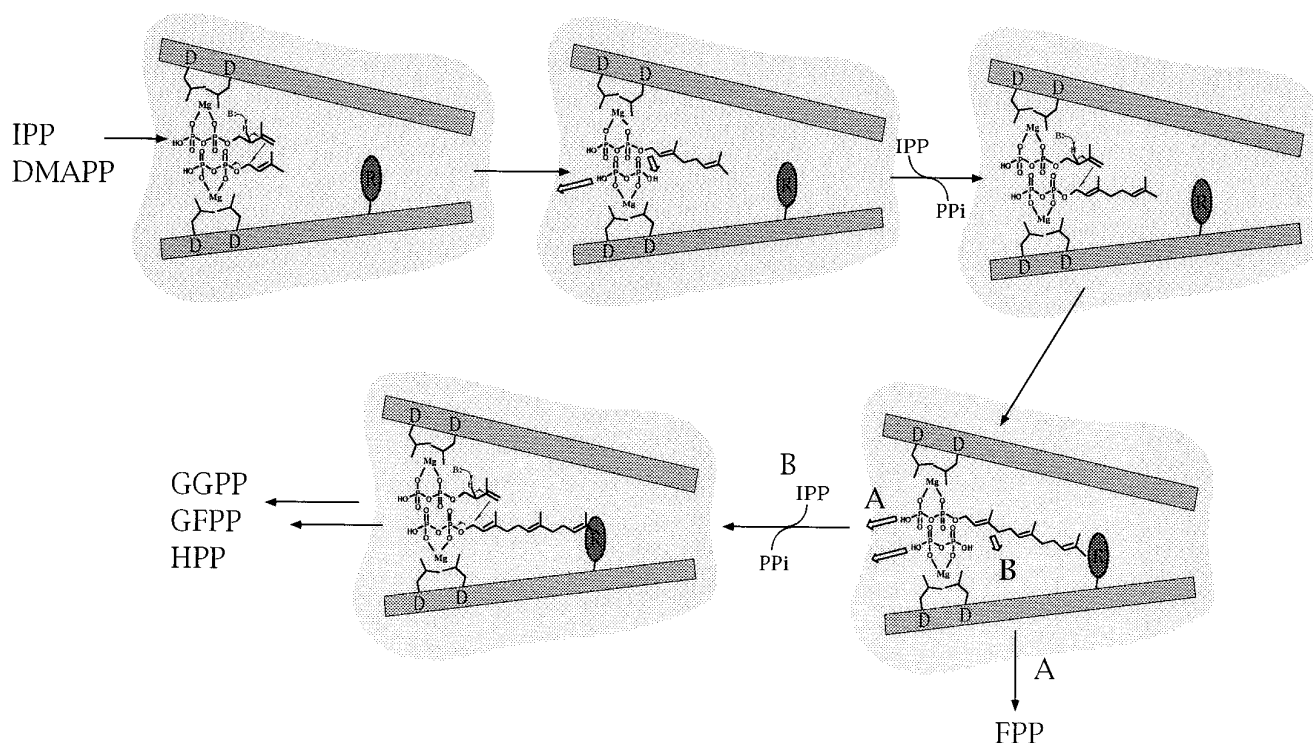


FIG. 4. **Simple model for consecutive condensations of IPP by FPP synthase.** First, FPP synthase accepts IPP and DMAPP. After two consecutive condensations of IPP, the ω -terminal of the formed FPP reaches the amino acid at the fifth position before the first aspartate-rich motif. If the side chain 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).

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-Å resolution by Tarshis *et al.* (11). The FPP synthase is composed of 13 α -helices jointed by connecting loops, and eight of them form a large central cavity. Two aspartate-rich motifs that are highly conserved among the pre-

nyltransferase 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. stearotheophilus* FPP synthases and indicated that the tyrosine-81 of *B. stearotheophilus* 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 ω -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. stearotheophilus* (20), and octaprenyl diphosphate synthase from *E. coli* (21). These enzymes have alanine at the position corresponding to position 81 of *B. stearotheophilus* 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,

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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REFERENCES

1. Poulter, C. D., and Rilling, H. C. (1982) in *Biosynthesis of Isoprenoid Compounds* (Spurgeon, S. L., and Porter, J. W., eds) pp. 161–224, John Wiley & Sons, New York
2. Chen, A., Kroon, P. A., and Poulter, C. D. (1994) *Protein Sci.* **3**, 600–607
3. Koyama, T., Obata, S., Osabe, M., Takeshita, A., Yokoyama, K., Uchida, M., Nishino, T., and Ogura, K. (1993) *J. Biochem.* **113**, 355–363
4. Ohnuma, S.-i., Nakazawa, T., Hemmi, H., Hallberg, A.-M., Koyama, T., Ogura, K., and Nishino, T. (1996) *J. Biol. Chem.* **271**, 10087–10095
5. Ohnuma, S.-i., Hirooka, K., Hemmi, H., Ishida, C., Ohto, C., and Nishino, T. (1996) *J. Biol. Chem.* **271**, 18831–18837
6. Ohnuma, S.-i., Koyama, T., and Ogura, K. (1991) *J. Biol. Chem.* **266**, 23706–23713
7. Ohnuma, S.-i., Suzuki, M., and Nishino, T. (1994) *J. Biol. Chem.* **269**, 14792–14797
8. Kunkel, T. A. (1985) *Proc. Natl. Acad. Sci. U. S. A.* **82**, 488–492
9. Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual*, 2nd Ed., Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
10. Fujii, H., Koyama, T., and Ogura, K. (1982) *Biochim. Biophys. Acta* **712**, 716–718
11. Tarshis, L. C., Yan, M., Poulter, C. D., and Sacchettini, J. C. (1994) *Biochemistry* **33**, 10871–10877
12. Lee, B., and Richards, F. M. (1971) *J. Mol. Biol.* **55**, 379–400
13. Chothia, C. (1976) *J. Mol. Biol.* **105**, 1–14
14. Hopp, T. P., and Woods, K. R. (1981) *Proc. Natl. Acad. Sci. U. S. A.* **78**, 3824–3828
15. Chou, P. Y., and Fasman, G. D. (1978) *Adv. Enzymol.* 45–148
16. Marrero, P. F., Poulter, C. D., and Edwards, P. A. (1992) *J. Biol. Chem.* **267**, 21873–21878
17. Joly, A., and Edwards, P. A. (1993) *J. Biol. Chem.* **268**, 26983–26989
18. Song, L., and Poulter, C. D. (1994) *Proc. Natl. Acad. Sci. U. S. A.* **91**, 3044–3048
19. Ashby, M. N., and Edwards, P. A. (1990) *J. Biol. Chem.* **265**, 13157–13164
20. Koike-Takeshita, A., Koyama, T., Obata, S., and Ogura, K. (1995) *J. Biol. Chem.* **270**, 18396–18400
21. Asai, K.-i., Fujisaki, S., Nishimura, Y., Nishino, T., Okada, K., Nakagawa, T., Kawamukai, M., and Matsuda, H. (1994) *Biochem. Biophys. Res. Commun.* **202**, 340–345

Carbohydrates, Lipids, and Other Natural Products:

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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