Identification of Significant Residues in the Substrate Binding Site of *Bacillus stearothermophilus* Farnesyl Diphosphate Synthase†

Tanetoshi Koyama,*,‡ Masaya Tajima,‡ Hiroaki Sano,‡ Takashi Doi,‡ Ayumi Koike-Takeshita,§ Shusei Obata,§ Tokuzo Nishino,‡ and Kyozo Ogura*,||

Department of Biochemistry and Engineering, Faculty of Engineering, Tohoku University, Aoba-ku, Sendai 980-77, Bio Research Laboratory, Toyota Motor Corporation, Toyota-cho 1, Toyota, Aichi 471-71, and Institute for Chemical Reaction Science, Tohoku University, Aoba-ku, Sendai 980-77, Japan

Received January 19, 1996; Revised Manuscript Received April 9, 1996⊗

ABSTRACT: Farnesyl diphosphate synthases have been shown to possess seven highly conserved regions (I−VII) in their amino acid sequences [Koyama *et al*. (1993) *J. Biochem. (Tokyo) 113*, 355−363]. Site-directed mutants of farnesyl diphosphate synthase from *Bacillus stearothermophilus* were made to evaluate the roles of the conserved aspartic acids in region VI and lysines in regions I, V, and VI. The aspartate at position 224 was changed to alanine or glutamate (mutants designated as D224A and D224E, respectively); aspartates at positions 225 and 228 were changed to isoleucine and alanine (D225I, D228A); lysine at position 238 was changed to either alanine or arginine (K238A, K238R). The lysines at positions 47 and 183 were changed to isoleucine and alanine (K47I, K183A), respectively. Kinetic analyses of the wild-type and mutant enzymes indicated that the mutagenesis of Asp-224 and Asp-225 resulted in a decrease of k_{cat} values of approximately 10^4- to 10^5-fold compared to the wild type. On the other hand, D228A showed a k_{cat} value approximately one-tenth of that of the wild type, and the K_m value for isopentenyl diphosphate increased approximately 10-fold. Both K47I and K183A showed K_m values for isopentenyl diphosphate 20-fold larger and k_{cat} values 70-fold smaller than the wild type. These results suggest that the two conserved lysines in regions I and V contribute to the binding of isopentenyl diphosphate and that the first and the second aspartates in region VI are involved in catalytic function. Aspartate-228 is also important for the binding of isopentenyl diphosphate rather than for catalytic reaction.

Prenyltransferases constitute a large family of enzymes that catalyze the sequential condensation of isopentenyl diphosphate (IPP)[1] with allylic diphosphates to produce prenyl diphosphates, which lead to more than 20 000 naturally occurring isoprenoid compounds (Poulter & Rilling, 1982). The prenyltransferase reaction (Figure 1) is very interesting from mechanistic viewpoints in that it is a repetition of stereospecific condensation of IPP with prenyl diphosphates to give products with discrete chain lengths and stereochemistries fixed by the specificities of individual enzymes (Ito *et al*., 1987; Ohnuma *et al*., 1991).

Farnesyl diphosphate (FPP) synthase [EC 2.5.1.10] is the most widely distributed prenyltransferase. It catalyzes the consecutive condensations of IPP with dimethylallyl diphosphate (DMAPP) and then with the resulting product, geranyl diphosphate (GPP), to produce FPP as the final product as shown in Figure 2. FPP is a key precursor for most of the isoprenoid compounds of physiological significance. Recently the three-dimensional structure of avian recombinant FPP synthase has been determined by Tarshis *et al*. (1994).

Comparison of the primary structures of a number of prenyltransferases from a wide range of organisms, from bacteria to human, revealed the presence of seven highly conserved regions, regions I−VII (Koyama *et al*., 1993). Figure 3 shows a typical alignment of the seven highly conserved regions for various prenyltransferases. Regions II and VI are the most remarkable in that they contain Asp-rich motifs LxxDDxxxxDxxxxRRG and GxxFQxxD-DxxD...GK, respectively, where x encodes any amino acid. These regions, designated by Ashby and Edwards (1990) as domains I and II, respectively, were thought to be binding sites for the diphosphate moieties in IPP and the allylic diphosphate substrates (Ashby *et al*., 1990). As pointed out in Figure 3, we propose that another highly conserved region, designated as region VIII, should be typical of prenyltransferases that catalyze the synthesis of prenyl diphosphate longer than C_{20}.

Several site-directed mutagenesis experiments have shown that most of the conserved Asp and Arg residues in the two regions are crucial for catalytic function. A number of mutant FPP synthases with markedly decreased k_{cat} values have been prepared, but only a few mutant enzymes showed significant changes in the K_m values for either the homoallylic substrate (IPP) or the allylic substrates (GPP and DMAPP). Substantially different results were reported by two groups. Song and Poulter (1994) reported that mutation at the first and second Asp residues, Asp^{240} and Asp^{241}, in region VI (domain II) of *Saccharomyces cerevisiae* FPP synthase

† This work was supported by Grants-in-Aid for Scientific Research, Nos. 07680620, 07555252, and Priority Areas No. 06240102 from the Ministry of Education, Science, Sports and Culture, Japan.

* Authors to whom correspondence should be addressed.

‡ Department of Biochemistry and Engineering, Faculty of Engineering, Tohoku University.

§ Bio Research Laboratory, Toyota Motor Corporation.

|| Institute for Chemical Reaction Science, Tohoku University.

⊗ Abstract published in *Advance ACS Abstracts,* June 15, 1996.

[1] Abbreviations: IPP, isopentenyl diphosphate; FPP farnesyl diphosphate; DMAPP, dimethylallyl diphosphate; GPP, geranyl diphosphate; PCR, polymerase chain reaction; Tris, trishydroxymethylaminomethane; TLC, thin-layer chromatography; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis.

S0006-2960(96)00137-7 CCC: $12.00

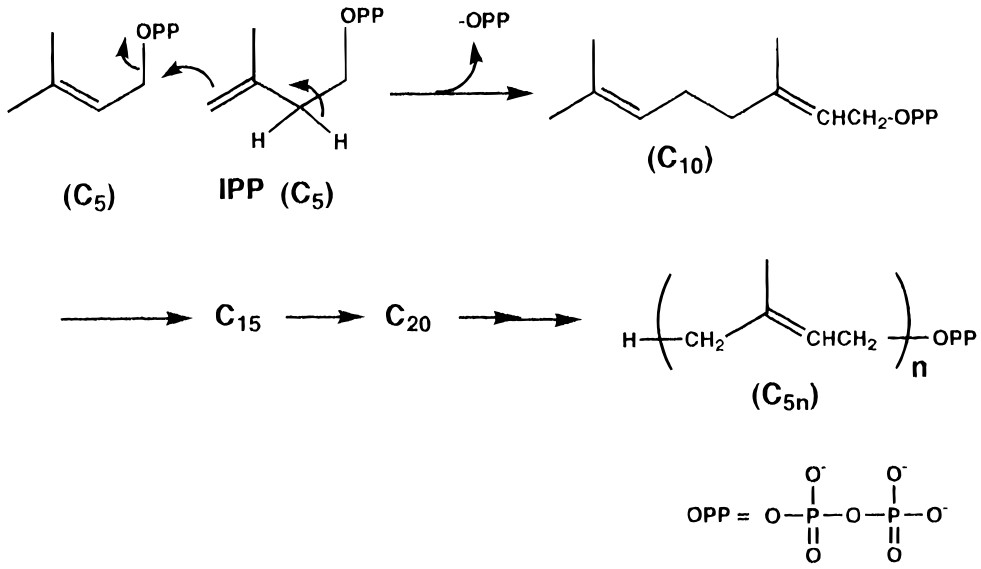

FIGURE 1: Reaction catalyzed by prenyltransferase.

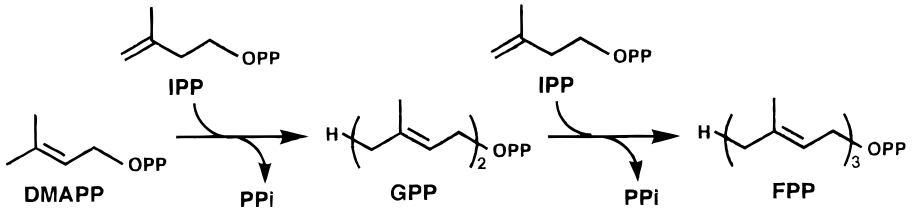

FIGURE 2: FPP synthase reaction.

drastically reduced the catalytic activity. Mutation at Asp[244], the third Asp in the same region, resulted in a reduction in k_{cat} by a factor of 16 and a 5-fold increase in K_m for IPP. On the other hand, Marrero *et al*. (1992) reported that mutation at the first Asp residue in region VI of rat liver enzyme drastically reduced the catalytic activity and increased the K_m value for IPP (26-fold) but mutation at the second Asp resulted in a reduction in k_{cat} by a factor of 8 but no change in K_m values. However, replacement of the third Asp in region VI of the liver enzyme with Glu had almost no effect on either k_{cat} or K_m values. Joly and Edwards (1993) reported in their extensional work with the liver enzyme that the conserved Asp and Arg residues in region II (domain I) are critical for efficient enzyme catalysis, since these mutations resulted in heavy decreases in V_{max} without remarkable changes in the K_m for IPP or GPP. Song and Poulter (1994) also investigated a series of site-directed mutants on these highly conserved Asp and Arg residues in the region II of yeast recombinant FPP synthase. As a result, mutations of the conserved charged residues in region II to noncharged residues, including Asp to Ala and Arg to Gln mutations drastically reduced the catalytic activity of the yeast enzyme.

Recently we showed that substitution of Glu for the highly conserved Gln in the conserved motif in region VI of *Bacillus stearothermophilus* enzyme caused 25- and 2-fold increases in the K_m values for DMAPP and GPP, respectively (Koyama *et al*., 1995).

Blanchard and Karst (1993) isolated the mutant gene *erg20-2* from a yeast strain that is defective in FPP synthase but able to excrete geraniol. They have shown that the unusual property of excreting geraniol is attributed to a one-point mutation resulting in a substitution of Glu for Lys-197 of *S. cerevisiae* FPP synthase (corresponding to Lys-183 in region V of the *B. stearothermophilus* enzyme). It is interesting to investigate the detailed relationship between the point mutation and the abnormal excretion of geraniol.

In order to better understand the role of the Lys residues conserved in region V, in region I, and at the downstream within region VI, we separately mutated each of the conserved Lys residues. We also made some substitutions of the three conserved Asp residues in region VI to investigate the roles of the regions more collectively.

We report here the finding that the Lys residues conserved in the regions I and V as well as one of the conserved Asp residues in region VI are involved in the substrate binding sites of FPP synthase.

EXPERIMENTAL PROCEDURES

Materials. [1-[14]C]IPP (1.95 GBq/mol) was purchased from Amersham Corp. Nonlabeled IPP, DMAPP, and GPP were the same preparations as used in the previous work (Koyama *et al*., 1994). Restriction enzymes and other DNA-modifying enzymes were purchased from Takara Shuzo Co., Ltd. (Ohtsu, Japan) and Toyobo Co., Ltd. (Osaka, Japan), unless otherwise stated.

Site-Directed Mutagenesis. Site-directed mutagenesis was conducted using either the procedure by Kunkel *et al*. (1987) or the polymerase chain reaction (PCR).

The single-stranded DNA template of pEX47 (Koyama *et al*., 1994) was isolated with a helper phage, M13KO7 (Sambrook *et al*., 1989). The mutagenic oligonucleotides employed for the mutagenesis were 5′-CCTTTCAAAT-TCGCGCTGATATTCTCGATAT-3′ (for D224A, mismatched base is underlined); 5′-CTTTCAAATTCGCGAA-GATATTCTCGATATT-3′ (for D224E); 5′-TTTCAAA--

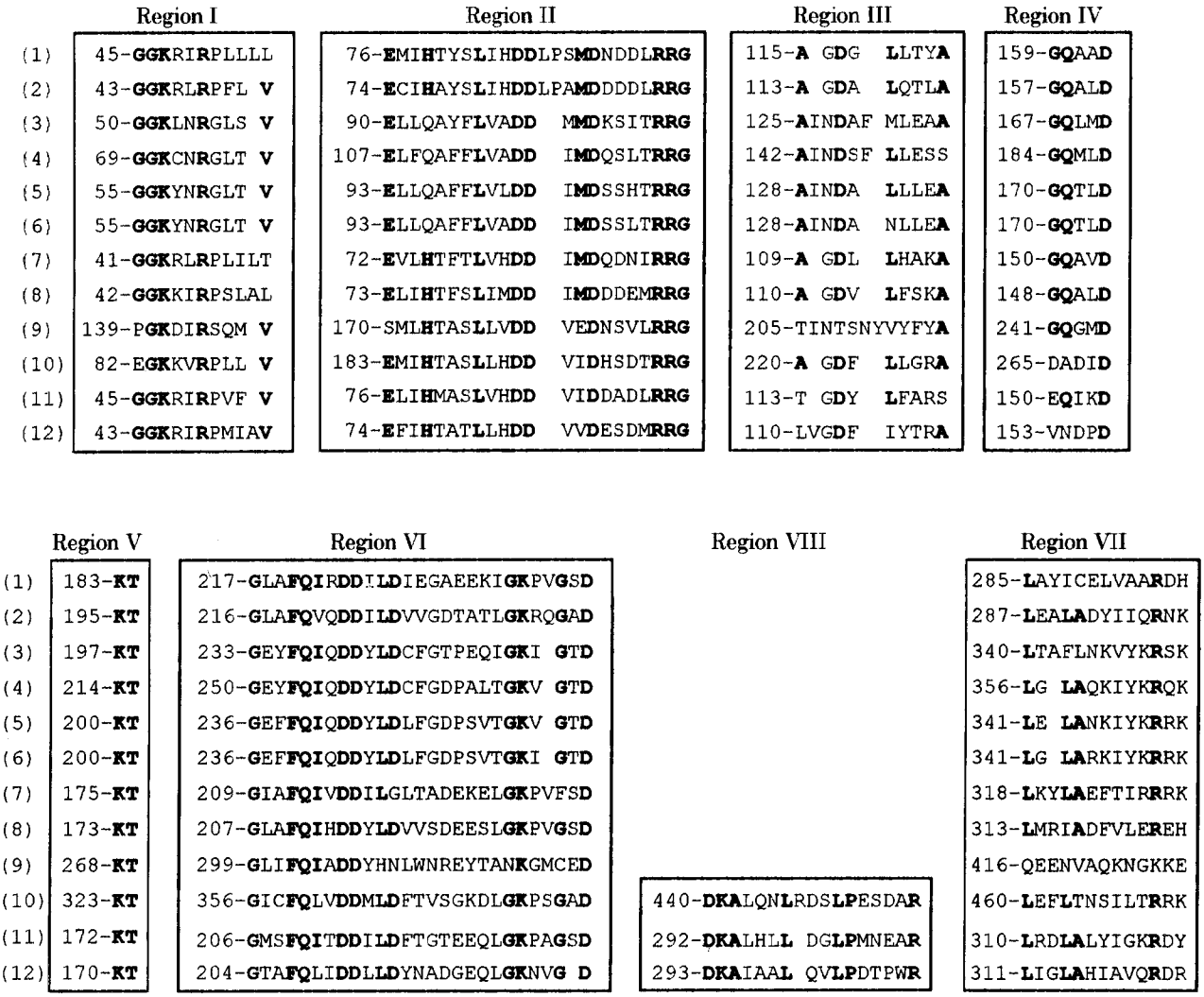

FIGURE 3: Amino acid sequence alignment of highly conserved regions for various prenyltransferases. The optimum amino acid alignment in regions I−VII of FPP synthase from (1) *B. stearothermophilus* (Koyama *et al*., 1993), (2) *E. coli* (Fujisaki *et al*., 1990), (3) *S. cerevisiae* (Anderson *et al*., 1989), (4) chicken (Chen *et al*., 1994), (5) rat (Clarke *et al*., 1987), and (6) human (Wilkin *et al*., 1990); geranylgeranyl diphosphate synthase from (7) *Sulfolobus acidocaldarius* (Ohnuma *et al*., 1994), (8) *Methanobacterium thermoautotrophicum* (Chen *et al*., 1994), and (9) *Neurospora crassa* (Carattoli *et al*., 1991); hexaprenyl diphosphate synthase from (10) *S. cerevisiae* (Ashby & Edwards, 1990); heptaprenyl diphosphate synthase from (11) *B. stearothermophilus* (Koike-Takeshita *et al*., 1995); and octaprenyl diphosphate synthase from (12) *E. coli* (Jeong *et al*., 1993) are shown. Numbers attached to the left of the sequences indicate the positions of the first amino acid displayed. Typical amino acids conserved in the enzymes are shown in boldface. Region VIII, which is located between regions VI and VII, can be found only in the enzymes that catalyze the synthesis of prenyl chains longer than C_{20}.

TTCGCGATATTATTCTCGATATTGAAG-3′ (for D225I); 5′-GAAGAAATCGGCGCGCCGGTCGGCAGCGA-3′ (for K238A); 5′-GAAGAAAAAATCGGCCGGCCGGTCGGC-AGCGA-3′ (for K238R); 5′-TGGAGGCCGGCGGCATTC-GAATCCGTCCGTTG-3′ (for K47I); and 5′-TACAT-TCATCGGCATGCAACCGGGAAAATGCT-3′ (for K183A). The introduction of mutation was confirmed by sequencing the whole nucleotide sequence using the dideoxy chain-termination method (Sanger et al., 1977) with a DNA sequencer (Applied Biosystems, model 373A).

For D228A, PCR was carried out with a mutagenic sense primer 5′-AAATTCGCGATGATATTCTCGCTATTGAA-GGGG-3′ (mismatched base is underlined) and an anti-sense primer 3′-CCGCCTACTCTCTTCTAAAAGTCGG-5′ in a similar procedure as described in our recent paper (Koyama et al. 1995).

Overproduction and Purification of FPP Synthase Mutants. The procedures employed for the overproduction and purification of FPP synthase mutants, D224A, D224E, D225I, D228A, K47I, K183A, K238R, and K238A, as well as the wild-type enzyme, were essentially similar to that previously reported by us (Koyama *et al*., 1993). The mutated enzymes were purified according to the procedure described in our previous report including heat treatment at 55 °C for 60 min followed by two steps of chromatography.

FPP Synthase Assay. The enzyme activity was measured by determination of the amount of [1-^{14}C]IPP incorporated into butanol-extractable polyprenyl diphosphates. In a standard assay procedure, the incubation mixture contained, in a final volume of 1.0 mL, 50 mM Tris-HCl buffer, pH 8.5, 50 mM $MgCl_2$, 50 mM NH_4Cl, 50 mM 2-mercaptoethanol, 6.55 μg of bovine serum albumin, 25 μM DMAPP or GPP, 50 μM [1-^{14}C]IPP (specific activity, 37 MBq/mol), and a suitable amount of enzyme. After incubation at 55 °C for 15 min, 2 mL of saturated NaCl solution was added and the reaction products were extracted with 1-butanol saturated with water. An aliquot of the butanol layer was counted for radioactivity.

For kinetic studies with the wild-type enzyme the concentrations of allylic substrate DMAPP/GPP or [1-^{14}C]IPP

Table 1: Kinetic Parameters of Wild-Type and Mutant FPP Synthases of *B. stearothermophilus*[a]

FPP synthase	K_m(IPP)[b] (μM)	K_m(GPP) (μM)	K_m(DMAPP) (μM)	$k_{cat} \times 10^4$ [c] (s^{-1})	k_{rel} [d]
wild-type	16.2 ± 1.2	9.7 ± 0.4	13.3 ± 1.7	110 000 ± 9700	1
D224A (VI)[e]	16.4 ± 1.7	2.7 ± 0.2	12.2 ± 2.2	7.9 ± 0.7	7.2×10^{-5}
D224E (VI)	17.7 ± 0.1	3.3 ± 0.4	22.3 ± 3.0	11.3 ± 0.4	1.0×10^{-4}
D225I (VI)	16.2 ± 0.1	5.2 ± 0.6	9.4 ± 3.0	2.1 ± 0.1	1.9×10^{-5}
D228A (VI)	160 ± 30	8.6 ± 0.7	77.9 ± 0.2	9800 ± 600	8.9×10^{-2}
K238A (VI)	70.4 ± 3.8	7.8 ± 0.1	14.1 ± 1.7	3790 ± 10	3.4×10^{-2}
K238R (VI)	57.9 ± 3.1	8.9 ± 0.1	11.4 ± 1.3	4260 ± 20	3.9×10^{-2}
K47I (I)	340 ± 63	4.8 ± 0.4	13.7 ± 1.5	1500 ± 5	1.4×10^{-2}
K183A (V)	303 ± 74	11.8 ± 3.3	ND[f]	1600 ± 6	1.5×10^{-2}

[a] Each value represents the mean ± SD of two determinations. [b] For the reaction with GPP. [c] k_{cat} was calculated from the V_{max} value for the reaction with IPP and GPP. [d] Relative k_{cat} value to that of wild type. [e] Region containing the indicated mutation. [f] Not determined.

(37 MBq/mol) were 2.5, 5.0, 10, 20, 30, 40, or 60 μM, while the other substrate [1-^{14}C]IPP or DMAPP/GPP was kept constant at 200 or 500 μM, respectively. The concentration of the enzyme was 13.5 ng/mL, and the incubation period was 10 min. With the mutant enzymes, the concentrations of varied substrates were 2.5, 5.0, 10, 20, 30, 40, or 60 μM except that for D228A, K47I, and K183A the concentrations of IPP were 100, 150, 200, 250, 300, and 350 μM, while the fixed substrate, [1-^{14}C]IPP or DMAPP/GPP, was kept at 50 or 500 μM, respectively, except that for D228A, K47I, and K183A the fixed substrate, [1-^{14}C]IPP or DMAPP/GPP, was 1 mM or 500 μM, respectively. The protein concentrations of the mutant enzymes, D224A, D224E, D225I, D228A, K238R, K238A, K47I, and K183A were 32.8, 16.1, 42.1, 1.3, 0.5, 7.5, 0.39, and 0.74 μg/mL, respectively, and the incubation periods were 30, 15, 60, 10, 15, 15, 15, and 15 min, respectively.

Product Analysis. After the enzymatic reaction at 55 °C for 60 min, the radioactive prenyl products in the reaction mixture were hydrolyzed to the corresponding alcohols with potato acid phosphatase according to our method (Koyama et al., 1985). The alcohols were extracted with pentane and analyzed by TLC on reversed phase LKC-18 (Whatman) in a solvent system of acetone/water (4:1). The positions of authentic standard alcohols were visualized with iodine vapor, and the distribution of radioactivity was determined by autoradiography. The TLC plates were exposed on a Fuji imaging plate at room temperature for 1 day, and then the imaging plate was analyzed with a Fuji BAS 1000 Mac bioimage analyzer. The relative amounts of the FPP and GPP produced were calculated from the intensities of the PSL (photostimulated luminescence) on the imaging plate.

RESULTS

Production of FPP Synthase Mutants. Figure 3 shows the amino acid sequence alignment of the seven highly conserved regions of various prenyltransferases. Region VI contains a characteristic motif, GxxFQ(I or L, V)xDDx(L or H)(D or G, N)xxxxxxxx(G or N)Kxx(G or F, C)xD, where x indicates any amino acid. In order to investigate the role of the conserved amino acids in this motif, site-directed mutagenesis studies were carried out with the thermostable FPP synthase of *B. stearothermophilus*. The conserved Asp at position 224 was changed to an aliphatic or the other acidic residue (D224A or D224E, respectively). The second and the third Asp residues at positions 225 and 228 were replaced with aliphatic amino acids, Ile and Ala (D225I, D228A), respectively. Lys at position 238 was changed to either aliphatic or another basic residue (K238A or K238R, respectively). In addition, two other fully-conserved Lys residues in other regions, Lys-47 in region I and Lys-183 in region V, were changed to aliphatic amino acids, Ile and Ala (K47I and K183A), respectively. Overproduction and subsequent purification of the mutant enzymes as well as the wild-type enzyme were carried out according to similar procedures including heat treatment at 55 °C for 60 min followed by two steps of chromatography (Koyama et al., 1993). SDS−PAGE analyses by staining with Coomassie Brilliant Blue showed that the purities of these mutant enzymes were more than 95%.

Kinetic Analysis of FPP Synthase Mutants. Kinetic parameters of the purified FPP synthase mutants are listed in Table 1. The mutagenesis of Asp-224 and Asp-225, including even the D224E mutagenesis, resulted in approximately 10^5-10^4 decrease in catalytic activity. Hence, it was necessary to substantially increase both the enzyme concentrations and incubation times to measure reliable values of reaction rates for these less reactive mutant enzymes. These facts indicate that both of the first and the second Asp residues in the DDxxD motif in region VI are critical for catalytic function. These three mutant enzymes, D224A, D224E, and D225I, however, showed comparable Michaelis constants for IPP to that of the wild type, and the values for GPP decreased. On the other hand, D228A showed a 10-fold larger K_m for IPP than the wild type, while it showed a comparable K_m value for GPP. The k_{cat} value of D228A is approximately one-tenth that of the wild type, indicating that the third Asp in the DDxxD motif is important for the binding of the homoallylic substrate, IPP, rather than for the catalytic reaction.

Substitutions of Ala and Arg for Lys-238 resulted in about 30- and 20-fold decreases of k_{cat} values, respectively, and they both caused 3−4-fold increases of K_m values for IPP. These facts imply that the conserved Lys residue downstream site within region VI contributes to the binding of IPP as well as to the catalytic reaction by this enzyme though the contribution to catalytic efficiency is much less important than those of the Asp residues at 224 and 225.

Both K47I and K183A showed considerable levels of FPP synthase activity, though their k_{cat} values were approximately 70-fold lower than that of the wild-type enzyme. Neither K47I nor K183A showed a significantly changed K_m value for GPP. In contrast, both of them showed markedly increased K_m values for IPP. These facts indicate that both Lys residues in regions I and V play important roles in the binding of IPP and that they are not essential for catalytic reaction. The K_m values for IPP of these mutants are the

Table 2: Relative Amount of Products of FPP Synthase Reactions by Mutated Enzymes[a]

FPP synthase	product distribution under low concn of IPP		product distribution under standard concn of IPP	
	GPP/FPP	conversion[g]	GPP/FPP	conversion[g]
wild-type	0.12[b]	74.5	0.02[c]	51.1
D224A	ND[f]	8.6	0.01[d]	5.8
D224E	ND	1.6	0.02[d]	1.2
D225I	ND	4.2	0.02[d]	2.8
D228A	0.14[e]	69.5	0[d]	59.5
K238A	0.31[b]	7.5	0.19[c]	8.8
K238R	0.23[b]	8.5	0.09[c]	9.9
K47I	1.56[b]	9.6	0.06[c]	7.2
K183A	1.22[b]	16.8	0.18[c]	13.1

[a] After incubation at 55 °C for 60 min, the prenyl diphosphate products were hydrolyzed with acid phosphatase, and then the radioactive alcohols were analyzed by TLC as described in Experimental Procedures. The relative amounts of the FPP and GPP produced were calculated from the intensities of PSL (photostimulated luminescence) on imaging plates with the bioimage analyzer. The enzymatic reactions were carried out with the concentrations of 25 μM DMAPP and [b]*0.5*, [c]*25*, [d]*50*, or [e]*1* μM of [1-^{14}C]IPP. [f] Not determined, because of the extremely low catalytic activities of the mutated enzymes. [g] Percentage of [1-^{14}C]IPP converted to products.

largest of all reported so far for the mutant enzymes derived from the *B. stearothermophilus* FPP synthase.

Product Analysis of FPP Synthase Mutants. After enzymatic reaction with GPP and [1-^{14}C]IPP as substrates, the radioactive prenyl diphosphates were hydrolyzed to the corresponding alcohols. TLC analyses of the alcohols indicated that all of the mutant enzymes catalyzed exclusive synthesis of FPP though the production rate varied individually (data not shown). When DMAPP was employed as the allylic primer substrate instead of GPP, a small amount of GPP was produced in addition to FPP. Table 2 shows the relative amounts of GPP and FPP produced from DMAPP and IPP in the reaction catalyzed by the mutant enzymes. When the initial concentration of IPP was the same as that of the standard conditions, *i.e*., the same as that of DMAPP, only a small amount of GPP was accumulated in the reaction catalyzed by the wild type. When the concentration of IPP was reduced to one fiftieth that of DMAPP, the product distribution ratio of GPP/FPP became more than one in both of the cases of K47I and K183A as shown in Figure 4. Under the same conditions the distribution ratio in the case of the wild type was at most 0.12.

DISCUSSION

In view of the finding that FPP synthase assay with a crude extract of a mutant strain of yeast gave GPP in preference to FPP, Blanchard and Karst (1993) assumed that the Lys-197 to Glu mutation might decrease the affinity of the enzyme for GPP, thereby causing a release of GPP. This is interesting in light of our observations that both K47I and K183A showed markedly increased K_m values for IPP and that GPP tends to accumulate in the reactions by these mutant enzymes. It is noteworthy that K47I and K183A resemble each other in both kinetic parameters and product distributions. These results raise the possibility that the increase of accumulation of GPP in the reaction catalyzed by the yeast mutant enzyme might also be due to their decrease in affinity of the binding site for IPP rather than for GPP.

On the other hand, D228A, K238A and K238R also showed 9.9-, 3.6-, and 4.3-fold increased K_m values for IPP,

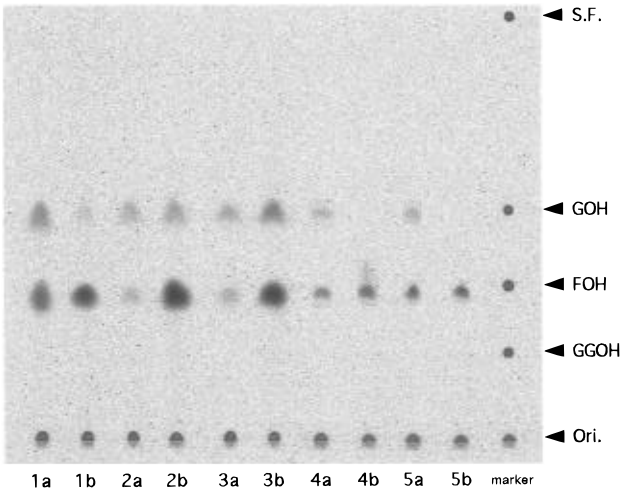

FIGURE 4: TLC radiochromatograms of the product alcohols of wild-type and mutant FPP synthase reactions with DMAPP and IPP as substrates. After enzymatic hydrolysis the product alcohols were analyzed as described in Experimental Procedures. Arrowheads indicate the positions of authentic prenols: GOH, geraniol; FOH, (*all-E*)-farnesol; GGOH, (*all-E*)-geranylgeraniol; ori., origin; S.F., solvent front. (a) Reaction with 0.5 μM [1-^{14}C]-IPP and 25 μM DMAPP; (b) reaction with 25 μM [1-^{14}C]IPP and 25 μM DMAPP. (1) Wild type, 13.5 μg; (2) K47I, 0.39 μg; (3) K183A, 0.74 μg; (4) K238A, 7.0 μg; (5) K238R, 7.4 μg.

respectively, compared to the wild type. However, the accumulation of GPP was at most 25% even when reduced concentrations of IPP were used. These facts indicate that substitution of Asp-228 or Lys-238 does not promote the release of GPP that is synthesized from DMAPP and IPP. Marrero et al. (1992) reported that the replacement of the third Asp in region VI with Glu did not affect the catalytic activity at all, showing there is some flexibility to allow even Ala to substitute for the Asp residue. However, the carboxylate moiety at this position must contribute to the binding with IPP because the K_m value for IPP of D228A is 10-fold higher than that of the wild type. These results suggest that the way of binding of IPP to Lys-47 and/or Lys-183 is somewhat different from that of Asp-228 and/or Lys-238 in region VI. Because the K_m values for IPP of D228A, K238A, and K238R are not so high as those of K47I and K183A, their decrease in affinity for IPP dose not result in GPP accumulation. This might be due to a similar effect of mutation to those observed for region VII mutants in which small increases of the K_m values for IPP were estimated for this bacterial enzyme and for the yeast enzyme by Koyama *et al*. (1994) and Song and Poulter (1994), respectively. It is noteworthy that the mutation in the second Asp residue resulted in the largest decrease in k_{cat} value among the mutant enzymes examined in this study. In this respect that bacterial enzyme is more similar to the yeast enzyme (Song & Poulter, 1994) than to the rat enzyme (Joly & Edwards, 1993). As the relative k_{cat} value to that of wild type, k_{rel} of D225I is comparable to that of F220A (Koyama *et al*., 1995), these residues seem to have the most important roles in the catalytic reaction.

It is noticeable that all of the mutants examined in this study except K183A had smaller K_m values for GPP than that of wild type. Especially, those of D224A, D224E, and D225I showed 2−3-fold decrease in the K_m value for GPP. However, most of the K_m values for DMAPP were comparable to that of wild type or rather increased. It is most

remarkable that D228A showed no change for the K_m for GPP but showed a 6-fold increase for DMAPP. A similar tendency has been reported on the mutants, F220A and Q221E, of this bacterial enzyme (Koyama *et al*., 1995). These facts suggest that the binding affinity of the C_{10}-allylic substrate (GPP) is distinct from that of the C_5-allylic substrate (DMAPP). This may be explained by assuming that the affinity for GPP is dominated by a strong interaction between its long hydrocarbon chain and nearby hydrophobic amino acid residues which probably form a cluster capable of accommodating various alkyl chains of proper size and shape, as suggested in our previous studies on substrate specificity (Ogura *et al*., 1970; Nishino *et al*., 1971, 1972).

Tarshis *et al*. (1994) have reported the three-dimensional structure of avian FPP synthase. Although the avian enzyme is larger by 70 amino acid residues and shows a 25% amino acid sequence identity with the *B. stearothermophilus* enzyme (Chen *et al*., 1994), the relative positions of the seven conserved regions are similar in all FPP synthases from prokaryotic and eukaryotic cells (Koyama *et al*., 1993). Thus, it is reasonable to assume that the active site of the bacterial enzyme has a three-dimensional structure similar to that of the avian enzyme. According to the structure of the chicken enzyme (Tarshis *et al*., 1994), Lys-71 and Lys-214, which correspond to Lys-47 and Lys-183 of the *B. stearothermophilus* enzyme, respectively, are located in the middle of the side wall inside a large deep cleft that forms the putative substrate binding cavity. The two Lys residues in regions I and V appear to reside in some proximity to each other in the cleft. The last Asp residue in the GxxFQxxDDxxD motif in region VI might be involved in forming a triad of IPP binding sites in collaboration with the two Lys residues.

REFERENCES

Anderson, M. S., Yargar, J. G., Burck, C. L., & Poulter, C. D. (1989) *J. Biol. Chem. 264*, 19176−19184.

Ashby, M. N., & Edwards, P. A. (1990) *J. Biol. Chem. 265*, 13157−13164.

Ashby, M. N., Spear, D. H., & Edwards, P. A. (1990) in *Molecular Biology of Atherosclerosis*: *Twentieth Steenbock Symposium* (Attie, A., Ed.) pp 27−34, Elsevier, New York.

Blanchard, L., & Karst, F. (1993) *Gene 125*, 185−189.

Carattoli, A., Romano, N., Ballario, P., Morelli, G., & Macino, G. (1991) *J. Biol. Chem. 266*, 5854−5859.

Chen, A., Kroon, P. A., & Poulter, C. D. (1994) *Protein Sci. 3*, 600−607.

Clarke, C. F., Tanaka, R. D., Svenson, K., Wamsley, M., Fogelman, A. M., & Edwards, P. A. (1987) *Mol. Cell. Biol. 7*, 3138−3146.

Fujisaki, S., Hara, H., Nishimura, Y., Horiuchi, K., & Nishino, T. (1990) *J. Biochem.* (*Tokyo*) *108*, 995−1000.

Ito, M., Kobayashi, M., Koyama, T., & Ogura, K. (1987) *Biochemistry 26*, 4745−4750.

Jeong, J.-H., Kitakawa, M., Isono, S., & Isono, K. (1993) *DNA Sequence 4*, 59−67.

Joly, A., & Edwards, P. A. (1993) *J. Biol. Chem. 268*, 26983−26989.

Koike-Takeshita, A., Koyama, T., Obata, S., & Ogura, K. (1995) *J. Biol. Chem. 270*, 18396−18400.

Koyama, T., Fujii, H., & Ogura, K. (1985) *Methods Enzymol. 110*, 153−155.

Koyama, T., Obata, S., Osabe, M., Takeshita, A., Yokoyama, K., Uchida, M., Nishino, T., & Ogura, K. (1993) *J. Biochem.* (*Tokyo*) *113*, 355−363.

Koyama, T., Saito, K., Ogura, K., Obata, S., & Takeshita, A. (1994) *Can. J. Chem. 72*, 75−79.

Koyama, T., Tajima, M., Nishino, T., & Ogura, K. (1995) *Biochem. Biophys. Res. Commun. 212*, 681−686.

Kunkel, T. A., Roberts, J. D., & Zakour, R. A. (1987) *Methods Enzymol. 154*, 367−382.

Marrero, P. F., Poulter, C. D., & Edwards, P. A. (1992) *J. Biol. Chem. 267*, 21873−21878.

Nishino, T., Ogura, K., & Seto, S. (1971) *Biochim. Biophys. Acta 235*, 322−325.

Nishino, T., Ogura, K., & Seto, S. (1972) *J. Am. Chem. Soc. 94*, 6849−6853.

Ogura, K., Nishino, T., Koyama, T., & Seto, S. (1970) *J. Am. Chem. Soc. 92*, 6036−6041.

Ohnuma, S., Koyama, T., & Ogura, K. (1991) *J. Biol. Chem. 266*, 23706−23713.

Ohnuma, S., Suzuki, M., & Nishino, T. (1994) *J. Biol. Chem. 269*, 14792−14797.

Poulter, C. D., & Rilling, H. C. (1982) in *Biosynthesis of Isoprenoid Compounds* (Spurgeon, S. L., & Porter, J. W., Eds.) Vol. 1, pp 161−224, John Wiley and Sons, New York.

Sambrook, J., Fritsch, E. F., & Maniatis, T. (1989) *Molecular Cloning*: *A Laboratory Manual*, 2nd ed., Cold Spring Harbor Laboratory, Cold Spring Harbor, New York.

Sanger, F., Nicklen, S., & Coulson, A. R. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 5463−5467.

Song, L., & Poulter, C. D. (1994) *Proc. Natl. Acad. Sci. U.S.A. 91*, 3044−3048.

Tarshis, L. C., Yan, M., Poulter, C. D., & Sacchettini, J. C. (1994) *Biochemistry 33*, 10871−10877.

Wilkin, D. J., Kutsunai, S. Y., & Edwards, P. A. (1990) *J. Biol. Chem. 265*, 4607−4614.

BI960137V