Site-Directed Mutagenesis of the Conserved Residues in Component I of *Bacillus subtilis* Heptaprenyl Diphosphate Synthase†

Yuan-Wei Zhang, Xiao-Yuan Li, Hiroshi Sugawara, and Tanetoshi Koyama*

Institute for Chemical Reaction Science, Tohoku University, Katahira 2-1-1, Aoba-ku, Sendai 980-8577, Japan

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ABSTRACT: Heptaprenyl diphosphate synthase of *Bacillus subtilis* is composed of two dissociable heteromeric subunits, component I and component II. Component II has highly conserved regions typical of (*E*)-prenyl diphosphate synthases, but it shows no prenyltransferase activity alone unless it is combined with component I. Alignment of amino acid sequences for component I and the corresponding subunits of *Bacillus stearothermophilus* heptaprenyl diphosphate synthase and *Micrococcus luteus* B-P 26 hexaprenyl diphosphate synthase shows three regions of high similarity. To elucidate the role of these regions of component I during catalysis, 13 of the conserved amino acid residues in these regions were selected for substitution by site-directed mutagenesis. Kinetic studies indicated that substitutions of Val-93 with Gly, Leu-94 with Ser, and Tyr-104 with Ser resulted in 3−10-fold increases of K_m values for the allylic substrate and 5−15-fold decreases of V_{max} values compared to those of the wild-type enzyme. The three mutated enzymes, V93G, L94S, and Y104S, showed little binding affinity to the allylic substrate in the membrane filter assay. Furthermore, product analyses showed that D97A yielded shorter chain prenyl diphosphates as the main product, while Y103S gave the final product with a C_{40} prenyl chain length. These results suggest that some of the conserved residues in region B of component I are involved in the binding of allylic substrate as well as determining the chain length of the enzymatic reaction product.

Prenyltransferase is the generic name for a family of enzymes catalyzing the consecutive condensation of isopentenyl diphosphate (IPP)[1] with allylic diphosphates to give prenyl diphosphates of different chain length and stereochemistry in the biosynthetic pathway of isoprenoid compounds, which are the most structurally diverse family of molecules found in nature (*1*−*3*). These enzymes can be classified into four groups according to the mode of requirement for enzymatic activity and the chain length and geometry of the final product that is determined by the distinct specificity of each enzyme (*4*). Among these prenyltransferases, medium-chain (*E*)-prenyl diphosphate synthases are unusual because of their heteromeric structures, which distinguish them from the other classes of prenyl diphosphate synthases that are tightly coupled homodimers such as farnesyl (*5*), solanesyl (*6*), or undecaprenyl (*7*) diphosphate synthase.

Medium-chain (*E*)-prenyl diphosphate synthases catalyze the condensation of IPP with (*E*,*E*)-farnesyl diphosphate (FPP) to form medium-chain (*E*)-prenyl diphosphates with chain lengths of C_{30} or C_{35}, which are responsible for the biosynthesis of the side chain of menaquinones or ubiquinones of some microorganisms. Heptaprenyl diphosphate (HepPP, C_{35}) synthase from *Bacillus subtilis* has been shown to be composed of two dissociable components, I and II, neither of which has any catalytic activity unless they are combined (*8*). Similarly, two different proteins, components A and B, constituting hexaprenyl diphosphate (HexPP, C_{30}) synthase were separated and characterized from *Micrococcus luteus* B-P 26 (*9, 10*). Therefore, the two enzymes appear to have novel heterodimeric structures with subunits easily dissociable under physiological conditions. However, the two subunits of the thermophilic bacterium *Bacillus stearothermophilus* HepPP synthase seem to be associated with each other, because there is no tendency to separate into the two components I′ and II′ during the course of purification of the native enzyme from *B. stearothermophilus* cells (*11*). It has been suggested that the thermophilic nature of *B. stearothermophilus* HepPP synthase is obtained by its tightly associated heterodimeric structure (*12*).

During the past decade, the structural genes for many kinds of prenyltransferases that catalyze (*E*)-prenyl chain elongation have been cloned and characterized (*13*). Multiple alignments of the putative amino acid sequences of these (*E*)-prenyl chain elongation enzymes have shown the presence of seven highly conserved regions including two characteristic aspartate-rich DDXXD motifs, which have been shown to be essential for catalytic function in several site-directed mutagenesis studies of FPP synthases (*14*−*17*). Tarshis et al. (*18*) determined the crystal structure of avian recombinant FPP synthase to 2.6 Å resolution, which is the first three-

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* To whom correspondence should be addressed. Telephone: +81-22-217-5621. Fax: +81-22-217-5620. E-mail: koyama@icrs.tohoku.ac.jp.

[1] Abbreviations: BSA, bovine serum albumin; FPP, (*E*,*E*)-farnesyl diphosphate; HepPP, heptaprenyl diphosphate; HexPP, hexaprenyl diphosphate; IPP, isopentenyl diphosphate; IPTG, isopropyl-β-D-thiogalactopyranoside; LB, Luria−Bertani; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; TLC, thin-layer chromatography; Tris, tris(hydroxymethyl)aminomethane.

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dimensional structure of any prenyltransferase. In the crystal structure of the FPP synthase, most of the conserved regions are found in a large central cavity, which is assigned to be the catalytic site. On the basis of the highly conserved amino acid sequences typical of prenyltransferases, we have identified the genes encoding three medium-chain (*E*)-prenyl diphosphate synthases including HepPP synthases of *B. subtilis* (*19*) and *B. stearothermophilus* (*20*) and HexPP synthase of *M. luteus* B-P 26 (*21*), confirming that each of these medium-chain (*E*)-prenyl diphosphate synthases comprises two essential protein components (components I and II or components I′ and II′ for HepPP synthase from *B. subtilis* or *B. stearothermophilus*, respectively, and components A and B for HexPP synthase from *M. luteus* B-P 26). The deduced amino acid sequences show that the larger subunits (components II, II′, and B or GerC3, Heps-2, and Hexs-B) have highly conserved regions that are characteristic of (*E*)-prenyltransferases, while the smaller subunits (components I, I′, and A or GerC1, Heps-1, and Hexs-A) have no similarity to the protein sequences registered so far in protein databases (*19*). As each of these medium-chain (*E*)-prenyl diphosphate synthases essentially requires the coexistence of both subunits for its catalysis, it has been proposed that the larger subunit supplies substantial sites for substrate binding and catalysis, whereas the smaller one plays an auxiliary but essential role in catalytic function (*20*).

The two components of *B. subtilis* HepPP synthase (components I and II) are encoded by two cistrons in a gene cluster of the *gerC* operon (*19*). The studies on the dynamic interaction between the two dissociable components during catalysis indicated that the two components, allylic substrate FPP and Mg^{2+}, form a catalytically active complex which represents an intermediary state during catalysis (*22*). Furthermore, photoaffinity labeling experiments with an alkyl group analogue of FPP suggested that component I of the enzyme possesses a specific affinity for the hydrophobic portion of the allylic substrate (*22*).

Comparison of the deduced amino acid sequences of component I (GerC1) of *B. subtilis* HepPP synthase with the corresponding components (Heps-1 and Hexs-A) of *B. stearothermophilus* HepPP synthase and *M. luteus* B-P 26 HexPP synthase indicates that component I has 38% identity to Heps-1 but shows only 8% identity to Hexs-A. The conserved amino acid residues among these components were proposed to be important for catalytic function (*21*). To better understand the role of these conserved amino acid residues during catalysis, we selected 13 of the conserved residues in component I of *B. subtilis* HepPP synthase for substitutions by site-directed mutagenesis. This paper describes the elucidation of several residues in component I that participate in the substrate binding and chain length determination during the HepPP synthase reaction.

EXPERIMENTAL PROCEDURES

Materials and General Procedures. [1-^{14}C]IPP (1.95 TBq/mol) and [1-^{3}H]FPP (2.22 TBq/mmol) were purchased from Amersham. Nonlabeled IPP and FPP were synthesized according to the procedure of Davisson et al. (*23*). Restriction enzymes and other DNA-modifying enzymes were from Takara Shuzo Co., Ltd., and Toyobo Co., Ltd. *E. coli* K12 strain JM109 was used as the host for expression of the target gene regions. Restriction enzyme digestions, transformations, and other standard molecular biology techniques were carried out as described by Sambrook et al. (*24*). Bacteria were cultured in Luria−Bertani (LB) or M9YG medium (*25*). The two wild-type components of HepPP synthase of *B. subtilis* were overproduced in *E. coli* cells and purified separately as previously described (*22*). All other chemicals were of analytical grade.

Amino Acid Sequence Alignment. Multialignment of amino acid sequences was performed using *GENETYX* genetic information processing software (Software Development).

Site-Directed Mutagenesis. To introduce mutations in the structural gene, *gerC*1, for the component I protein of *B. subtilis* HepPP synthase, a *Sph*I/*Eco*RI DNA fragment from the clone pSH01 (*22*) was subcloned into pUC119 (Takara Shuzo), yielding pUHA01. Site-directed mutagenesis was conducted with the double-stranded DNA template of pUHA01 according to the protocol of the GeneEditor in vitro site-directed mutagenesis system (Promega). Mutagenic oligonucleotides (20−30 bases long) were designed to change the desired residue and generate plasmids (pUHAMUs; see Table 1). Introduction of the mutation was confirmed by sequencing whole nucleotide sequences using the dideoxy chain-termination method (*26*) with a DNA sequencer (LI-COR, model 4200).

Construction of Expression Vector Systems for Mutated Component I. Each of the mutated plasmids (pUHAMUs) was digested with *Sph*I, treated with T_4 DNA polymerase, and digested again with *Pst*I. The resulting fragment was ligated into pTrc 99A vector (Pharmacia), which was digested with *Nco*I, treated with T4 DNA polymerase, and digested with *Pst*I, to construct each of the expression plasmids, pHAEXs (Table 1).

Overproduction and Purification of Mutated Component I. Each of the expression plasmids was used for transformation of *E. coli* JM109, and the overnight culture of *E. coli* cells harboring the pHAEX plasmid in LB medium containing 50 μg/mL ampicillin was inoculated into a 250-fold volume of M9YG medium containing 50 μg/mL ampicillin. The cells were grown at 37 °C to an approximate A_{600} value of 0.6, and isopropyl-β-D-thiogalactopyranoside (IPTG) was added to a final concentration of 1 mM, and then the incubation was continued for an additional 3 h at 30 °C. Overproduction of the proteins was confirmed by sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE).

Each of the mutated components I overproduced in *E. coli* cells was purified essentially according to the purification procedure of the wild-type component I as described in our previous work (*22*), including renaturation of these proteins with 8 M urea, followed by stepwise dialyses and three chromatographic steps. The fractions of the mutated proteins were analyzed for purity by SDS−PAGE with Coomassie Brilliant Blue staining, and the fractions that showed more than 90% purity were used for further characterization. Protein concentrations were measured by the method of Bradford (*27*) with bovine serum albumin (BSA) as the standard.

HepPP Synthase Assay and Product Analysis. The enzyme activity was measured by determination of the amount of [1-^{14}C]IPP incorporated into butanol-extractable polyprenyl diphosphates. A standard assay mixture contained, in a final

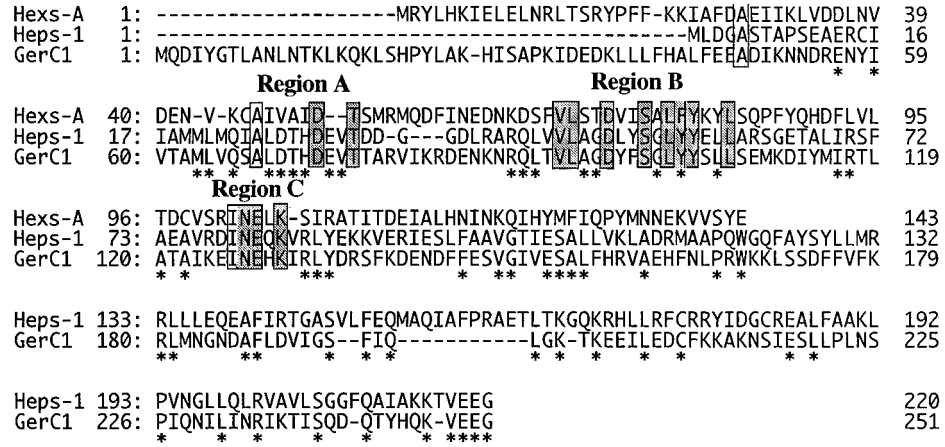

FIGURE 1: Amino acid sequence alignment of component I of *B. subtilis* HepPP synthase (GerC1) with the corresponding subunits of *B. stearothermophilus* HepPP synthase (Heps-1) and *M. luteus* B-P 26 HexPP synthase (Hexs-A). The fully conserved residues among the three subunits are boxed. Asterisks show the residues that are identical only between GerC1 and Heps-1. The residues mutated in this study were shaded.

volume of 0.3 mL, 0.30 μM [1-^{14}C]IPP (1.95 TBq/ mol), 25 mM Tris-HCl buffer, pH 8.5, 25 mM NH_4Cl, 10 mM 2-mercaptoethanol, 1 mM $MgCl_2$, 15 μM FPP, and a suitable amount of enzyme solution. After incubation at 37 °C for 20 min, the reaction products were immediately extracted with 1-butanol saturated with water, and the radioactivity in the butanol extract was measured with an Aloka LSC-1000 liquid scintillation counter.

The radioactive prenyl diphosphate products in the reaction mixture were hydrolyzed to the corresponding alcohols with potato acid phosphatase according to the method reported previously (*28*), and analyzed by reversed-phase thin-layer chromatography (TLC) plates (LKC-18, Whatman) with a solvent system of acetone/water (19/1). The positions of authentic standards were visualized with iodine vapor, and the distribution of radioactivity was detected. The TLC plates were exposed on a Fuji imaging plate at room temperature for 1 day, and then the plate was analyzed with a Fuji BAS 1000 Mac bioimage analyzer.

Measurement of the Dissociation Constants for FPP. The binding of the allylic substrate FPP to the HepPP synthase was measured essentially according to the method of Dolence et al. (*29*). To [1-^{3}H]FPP (18.5 GBq/mmol, 10−200 nM) in 25 mM Tris-HCl buffer, pH 8.5, containing 25 mM NH_4Cl, 10 mM 2-mercaptoethanol, and 1 mM Mg^{2+} (100 μL final volume) were added component I (13.8 nM) and component II (13.8 nM). The mixtures were incubated at room temperature for 15 min. An aliquot (10 μL) was removed from the tube and mixed with 6 mL of a liquid scintillation cocktail, Clear-Sol I (Nacalai Tesque Co., Ltd.), to determine the total radioactivity of the sample. The remainder of the mixture was transferred to a preequilibrated membrane filter, Microcon 10 (Amicon), and centrifuged at 14 000 rpm for more than 10 s until 10−15 μL of the solvent had passed through the membrane. The radioactivity in a 10 μL aliquot of the filtrate was counted to determine the concentration of unbound substrate. The radioactivity was corrected for membrane retention using a correction factor obtained from an identical experiment as described above except that two subunits were omitted. The unbound FPP concentration was subtracted from the substrate concentration employed to obtain the concentration of enzyme-bound FPP. The data were then fitted to a Scatchard plot and a standard binding equation (*30*) to obtain the K_d value of the substrate for the enzyme.

RESULTS

Sequence Alignments. The GENETYX-MAC (version 8.0) software was used to align amino acid sequences for the smaller subunits (GerC1, Heps-1, and Hexs-A) of the three medium-chain (*E*)-prenyl diphosphate synthases, HepPP synthases of *B. subtilis* and *B. stearothermophilus* and HexPP synthase of *M. luteus* B-P 26, respectively. This alignment, similar to the earlier report (*21*), revealed three highly conserved regions (A, B, and C), which might be important for catalytic function. We selected 13 of the conserved amino acid residues (shaded in Figure 1) among the 3 smaller subunits for substitution by site-directed mutagenesis.

Production of Site-Directed Mutants of Component I of B. subtilis HepPP Synthase. To examine the role of the conservative residues in regions A, B, and C, site-directed mutagenesis studies were carried out with component I of *B. subtilis* HepPP synthase. The hydropathic profile for this component protein indicated that regions A and C are hydrophilic, while region B is hydrophobic (data not shown). The conserved polar and charged residues in regions A and C were changed to aliphatic amino acids, Ala, Val, and Ile (D73A, T76V, N127A, E128V, and K130I), respectively, so that the regions were made less hydrophilic. The Leu at 94, 102, or 107 and the Tyr at 103 or 104 in region B were replaced with Ser (L94S, L102S, L107S, Y103S, and Y104S, respectively), which has a smaller and less hydrophobic side chain. The Asp at position 97 in region B was changed to an aliphatic residue, Ala (D97A). Val-93 and Ser-100 were replaced with Gly (V93G and S100G, respectively).

To construct expression systems for these mutants, oligonucleotide-mediated mutagenesis was conducted into plasmid pUHA01, in which *Sph*I and *Pst*I sites were previously introduced immediately upstream and downstream of the open reading frame of the *gerC*1 gene, respectively, to obtain the mutated plasmids pUHAMU. The *Sph*I/*Pst*I fragment of each of the pUHAMU plasmids was ligated into the *Nco*I/*Pst*I site of pTrc99A, and the expression plasmid pHAEX was obtained (Table 1).

After transformation of *E. coli* JM109 with each of the expression plasmids, the component I mutant was overpro-

Table 1: Site-Directed Mutations in Component I of *B. subtilis* HepPP Synthase

nucleotides	plasmid	region	amino acid	mutant name
GAT to GCT	pHAEX73	A	Asp73 to Ala	D73A
ACG to GTG	pHAEX76	A	Thr76 to Val	T76V
GTT to GGT	pHAEX93	B	Val93 to Gly	V93G
CTC to AGC	pHAEX94	B	Leu94 to Ser	L94S
GAT to GCT	pHAEX97	B	Asp97 to Ala	D97A
AGC to GGC	pHAEX100	B	Ser100 to Gly	S100G
CTG to TCG	pHAEX102	B	Leu102 to Ser	L102S
TAC to TCC	pHAEX103	B	Tyr103 to Ser	Y103S
TAC to TCC	pHAEX104	B	Tyr104 to Ser	Y104S
CTA to TCA	pHAEX107	B	Leu107 to Ser	L107S
AAC to GCC	pHAEX127	C	Asn127 to Ala	N127A
GAA to GTA	pHAEX128	C	Glu128 to Val	E128V
AAA to ATA	pHAEX130	C	Lys130 to Ile	K130I

Table 2: Kinetic Parameters for the Wild-Type and Mutated Enzymes[a]

	K_m(IPP) (μM)	K_m(FPP) (μM)	V_{max}[b] (nmol min^{-1} nmol^{-1})
wild-type	16.7 ± 1.8	7.2 ± 0.9	1100 ± 30
D73A	16.1 ± 1.2	6.4 ± 0.4	1248 ± 50
T76V	18.3 ± 1.0	8.4 ± 0.5	985 ± 15
V93G	22.6 ± 3.3	74.1 ± 10.2	70 ± 5
L94S	19.4 ± 2.5	50.8 ± 8.5	120 ± 8
D97A	15.6 ± 0.9	6.9 ± 1.0	1573 ± 25
S100G	14.2 ± 0.5	10.3 ± 2.0	860 ± 14
L102S	20.3 ± 4.6	7.2 ± 1.1	1320 ± 38
Y103S	18.8 ± 2.5	9.8 ± 1.5	1042 ± 18
Y104S	16.7 ± 2.0	20.0 ± 3.2	215 ± 9
L107S	17.5 ± 1.6	5.3 ± 0.3	990 ± 15
N127A	19.4 ± 3.0	8.2 ± 0.5	1110 ± 18
E128V	17.2 ± 1.6	5.6 ± 0.2	1250 ± 20
K130I	13.4 ± 0.8	7.2 ± 1.1	1080 ± 16

[a] The combination of the wild-type or mutated component I and an equimolar wild-type component II was used as the heteromeric enzyme for kinetic studies. [b] Nanomoles of IPP converted to products per minute by 1 nmol of enzyme (a combination of 1 nmol of component I and 1 nmol of component II).

duced and purified essentially according to the procedure for the wild-type component I (*22*). SDS−PAGE analyses by staining with Coomassie Brilliant Blue showed that the purities of these mutants were more than 90%.

Kinetic Analysis of HepPP Synthase Mutants. Enzyme activity of the purified component I mutant was determined by supplying the purified wild-type component II. Michaelis constants (K_m) for the allylic substrate FPP and homoallylic substrate IPP and V_{max} were determined for all mutated enzymes, and the results are listed in Table 2. All mutant enzymes showed Michaelis constants for the homoallylic substrate IPP comparable to that of the wild-type. Moreover, all five mutants with respect to regions A and C, D73A, T76V, N127A, E128V, and K130I, showed similar K_m values for FPP and V_{max} values compared to the wild-type. Three of the mutants in region B, V93G, L94S, and Y104S, however, had 5−15-fold lower V_{max} values and 3−10-fold higher K_m values for the allylic substrate FPP than those of the wild-type enzyme.

Dissociation Constants for FPP. Membrane filter assay was employed to determine the dissociation constants of the wild-type and three of the mutated HepPP synthases, V93G, L94S, and Y104S, for the allylic substrate FPP. The wild-type enzyme (a combination of the two wild-type components I and II) showed a typical affinity for FPP which

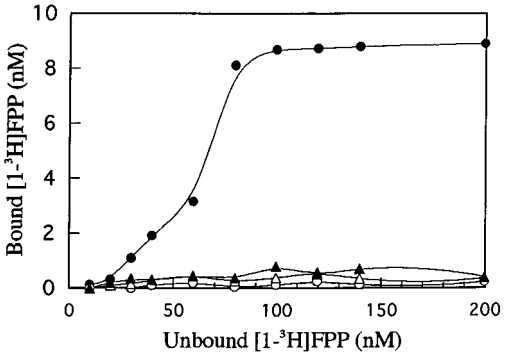

FIGURE 2: Plots of bound versus unbound FPP for determination of the dissociation constant for FPP with wild-type (●), V93G (○), L94S (△), or Y104S (▲).

increased dramatically up to saturation with an increase in the concentration of FPP. With 13.8 nM enzyme, duplicate measurements with [1-^{3}H]FPP at various concentrations yielded a dissociation constant (K_d) value of 60 ± 10 nM for FPP. In contrast, little binding of FPP was detected with the V93G, L94S, or Y104S mutants at concentrations of FPP up to 0.2 μM (Figure 2).

Product Analysis. After enzymatic reaction with FPP and [1-^{14}C]IPP as substrates, the radioactive prenyl diphosphate products were hydrolyzed to the corresponding alcohols. TLC analyses of the alcohols indicated that 11 mutated enzymes catalyzed the formation of HepPP as the ultimate product as well as some intermediate shorter chain prenyl diphosphates similar to those in the reaction catalyzed by the wild-type enzyme (data not shown). On the other hand, D97A and Y103S gave reaction products that have marked differences in chain length distribution from the wild-type enzyme (Figure 3). D97A produced larger amounts of shorter chain prenyl diphosphates, while Y103S gave octaprenyl diphosphate (C_{40}) as the final product by enzymatic prenyl chain elongation.

Heat Stability of Component I Mutants. Heat stabilities of the component I mutants were examined by analyzing their remaining activity with a supplement of the wild-type component II after heat treatment at various temperatures for 30 min. All of the mutants exhibited similar thermostabilities to that of the wild-type, retaining almost the original activities even after heating at 50 °C for 30 min (data not shown).

DISCUSSION

HexPP synthase and HepPP synthase constitute a group of prenyltransferases that catalyze the formation of medium-chain (*E*)-prenyl diphosphates, each of which has a heterodimeric structure. The larger subunit has highly conserved regions typical of all (*E*)-prenyltransferases; however, it shows no prenyltranferase activity by itself unless it is combined with the smaller one. The heterodimeric structures of these enzymes are distinctive from the other classes of (*E*)-prenyltransferases, which show catalytic functions by the homodimeric structures. Hence, the smaller subunits in the medium-chain (*E*)-prenyl diphosphate synthases confer unique properties on these enzymes as the combinations of the two subunits.

Several lines of evidence have indicated that the smaller subunit (component I) of *B. subtilis* HepPP synthase is

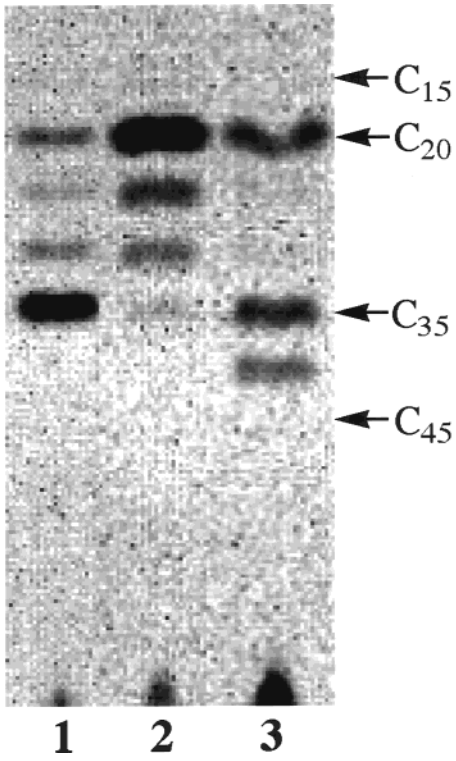

FIGURE 3: Product analysis of the reactions catalyzed by the wild-type and mutant enzymes. The alcohols obtained by enzymatic hydrolysis of the products formed by the incubation of [1-^{14}C]IPP and FPP with wild-type (lane 1), D97A (lane 2), or Y103S (lane 3) were analyzed by reversed-phase TLC as described under Experimental Procedures. The TLC plate was exposed on a Fuji imaging plate, and then the plate was analyzed with a Fuji BAS 1000 Mac bioimage analyzer. Arrowheads indicate the positions of authentic alcohols: C_{15}, (*E*,*E*)-farnesol; C_{20}, (*all-E*)-geranylgeraniol; C_{35}, (*all-E*)-heptaprenol; and C_{45}, (*all-E*)-nonaprenol.

involved in allylic substrate binding (*22*). We compared the primary structure of this component with the corresponding components (Heps-1 and Hexs-A) of *B. stearothermophilus* HepPP synthase and *M. luteus* B-P 26 HexPP synthase and found three conserved regions, A, B, and C. To elucidate the significance of the conserved residues in substrate binding and catalytic function, 13 kinds of mutated component I were prepared by oligonucleotide-mediated mutagenesis.

All these mutant enzymes, as combinations of the mutant components I and wild-type component II, showed comparable K_m values for the homoallylic substrate IPP to that of the wild-type enzyme, suggesting that none of the 13 conserved amino acid residues is involved in the binding of IPP. On the other hand, component II of *B. subtilis* HepPP synthase has two characteristic aspartate-rich DDXXD motifs, which have been shown to bind the diphosphate moieties of the homoallylic and allylic substrates through Mg^{2+} in the crystal structure of avian FPP synthase (*31*). In addition, no association between the two components of *B. subtilis* HepPP synthase to form a complex in the presence of Mg^{2+} and IPP was observed in our previous work (*22*). Hence, it is suggested that component I of the enzyme does not directly participate in the binding of the homoallylic substrate IPP.

As compared with the wild-type enzyme, three mutants in region B, V93G, L94S, and Y104S, show 3−10 times larger K_m values for the allylic substrate FPP and 5−15-fold lower V_{max} values. Furthermore, we examined the binding properties for FPP of the three mutants by adding the wild-type component II using the membrane filter assay. In comparison with the K_d value of the wild-type enzyme, 60 ± 10 nM, heavy decreases in the affinity for FPP were observed with V93G, L94S, and Y104S. These facts suggest that the binding affinity for FPP is affected by the substitutions of Val-93, Leu-94, and Tyr-104 with Gly and Ser, which have smaller and less hydrophobic side chains. Component I had been suggested to be involved in the binding of the hydrophobic prenyl tail of the allylic substrate FPP by our previous photoaffinity experiments, in that a photoreactive analogue of FPP, which contains a benzophenone group positioned at the tail of the prenyl chain, became covalently attached specifically to component I in the presence of component II (*22*). It is likely that the hydrophobic region B containing Val-93, Leu-94, and Tyr-104 of component I is located in a space that participates in the binding of the prenyl tail of the allylic substrate. Koyama et al. (*32*) have reported that an aromatic amino acid residue, Phe, which is located at the fifth position upstream to the second DDXXD motif in FPP synthase of *B. stearothermophilus*, is involved in the binding of allylic substrate and catalysis by exerting its aromatic hydrophobicity on a methyl group of DMAPP or/and hydrophobic aromaticity on a prenyl cation formed at the start of the catalytic reaction through a cation−π interaction. Thus, it is reasonable to propose that Tyr at position 104 in component I of *B. subtilis* HepPP synthase may participate in the binding of the allylic substrate and catalytic function of the enzyme by performing its aromaticity on the hydrophobic prenyl chain moiety to keep the binding of allylic substrate. Recently, the crystal structure of avian FPP synthase with allylic substrates bound has been analyzed (*31*), and it has been shown that allylic diphosphates bind through Mg^{2+} to the aspartates of the conserved Asp-rich motif (DDXXD) with the hydrocarbon tails of the ligands growing down the hydrophobic pocket. Though *B. subtilis* HepPP synthase is a heterodimer, component II has motifs similar to those of avian FPP synthase including the two DDXXD sequences. It is therefore reasonable to assume that component II has a tertiary structure similar to the subunit of avian FPP synthase. We predicted the secondary structure of component I of *B. subtilis* HepPP synthase according to the methods of Chou and Fasman (*33*) and Robson (*34*). In the secondary structure, region B of this component shows two antiparallel β-sheets that formed from residues 88−96 and 101−107, respectively, joined by a connecting loop composed of residues 97−100 (data not shown). Taken together, we propose a hypothetical model for the binding of the allylic substrate FPP of the two components as HepPP synthase (Figure 4). The diphosphate moiety of FPP seems to bind through Mg^{2+} to the DDXXD motif of component II, and the prenyl tail of FPP stretches into a hydrophobic pocket or wall containing Val-93, Leu-94, and Tyr-104 in region B of component I.

It is noteworthy that both D97A and Y103S gave reaction products that have significant differences in chain length distribution from the wild-type, though they showed comparable kinetic properties with those of the wild-type enzyme. D97A yielded shorter chain prenyl diphosphates as the main products, and Y103S gave an ultimate product with a C_{40} prenyl chain length. Recent reports (*31, 35*) showed that the aromatic amino acid residue that is located at the fifth

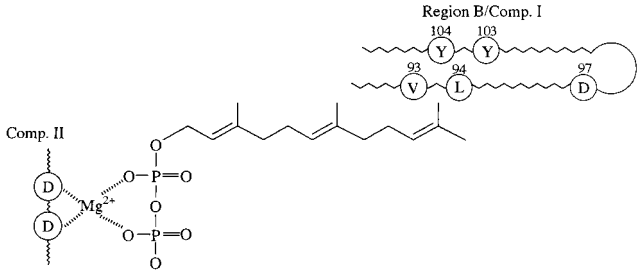

FIGURE 4: Hypothetical scheme for the binding of FPP between the two components of *B. subtilis* HepPP synthase. Comp. I, component I; Comp. II, component II.

position upstream of the first DDXXD motif in avian/*B. stearothermophilus* FPP synthase forms the floor of the putative allylic substrate binding pocket and is important for controlling product chain length. When this aromatic residue was replaced with Ala, Gly, or Ser having a smaller and more flexible side chain, the mutated FPP synthase could produce geranylgeranyl (C_{20}), geranylfarnesyl (C_{25}), and longer chain prenyl diphosphates. The reason that the replacements of Asp-97 and Tyr-103 with Ala and Ser led to altering chain length distribution may be explained by assuming that the substitutions result in some movement of the protein side chain and alteration of the size of the binding pocket for the growing isoprenoid chain.

The present studies revealed a subset of five residues in region B (Val-93, Leu-94, Asp-97, Tyr-103, and Tyr-104) that is involved in the binding of allylic substrate FPP and determining the chain length of reaction products. In contrast, none of the mutants with respect to regions A and C showed significantly changed kinetic properties, indicating that these replacements of the polar or charged residues with the aliphatic amino acids do not affect the binding of substrates and catalysis.

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