Chain Length Determination of Prenyltransferases: Both Heteromeric Subunits of Medium-Chain (*E*)-Prenyl Diphosphate Synthase Are Involved in the Product Chain Length Determination†

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Received June 7, 2000

ABSTRACT: Among prenyltransferases, medium-chain (*E*)-prenyl diphosphate synthases are unusual because of their heterodimeric structures. The larger subunit has highly conserved regions typical of (*E*)-prenyltransferases. The smaller one has recently been shown to be involved in the binding of allylic substrate as well as determining the chain length of the reaction product [Zhang, Y.-W., et al. (1999) *Biochemistry 38*, 14638−14643]. To better understand the product chain length determination mechanism of these enzymes, several amino acid residues in the larger subunits of *Micrococcus luteus* B-P 26 hexaprenyl diphosphate synthase and *Bacillus subtilis* heptaprenyl diphosphate synthase were selected for substitutions by site-directed mutagenesis and examined by combination with the corresponding wild-type or mutated smaller subunits. Replacement of the Ala at the fifth position upstream to the first Asp-rich motif with bulky amino acids in both larger subunits resulted in shortening the chain lengths of the major products, and a double combination of mutant subunits of the heptaprenyl diphosphate synthase, I-D97A/II-A79F, yielded exclusively geranylgeranyl diphosphate. However, the combination of a mutant subunit and the wild-type, I-Y103S/II-WT or I-WT/II-I76G, produced a C_{40} prenyl diphosphate, and the double combination of the mutants, I-Y103S/II-I76G, gave a reaction product with longer prenyl chain up to C_{50}. These results suggest that medium-chain (*E*)-prenyl diphosphate synthases take a novel mode for the product chain length determination, in which both subunits cooperatively participate in maintaining and determining the product specificity of each enzyme.

Prenyltransferases (so-called prenyl diphosphate synthases) catalyze the sequential head-to-tail condensation of isopentenyl diphosphate (IPP)[1] with allylic diphosphates to give linear prenyl diphosphates in the biosynthetic pathway of isoprenoid compounds. Although the chemical mechanisms of these condensation reactions are identical, there are a number of enzymes having different specificities with respect to the chain length and double bond stereochemistry of the products (*1*). One of the most interesting subjects on the catalytic mechanism of prenyltransferases is to understand how the individual enzymes determine their product chain lengths (*2*).

Medium-chain (*E*)-prenyl diphosphate synthases catalyze the condensation of IPP with (*E*,*E*)-farnesyl diphosphate (FPP) to form the 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 ubiquinones or menaquinones of some microorganisms. Among prenyltransferases, medium-chain (*E*)-prenyl diphosphate synthases are exceptional because of their heteromeric structures, which distinguish them from other classes of prenyltransferases composed of tightly coupled homodimers (*3*). Hexaprenyl diphosphate (HexPP, C_{30}) synthase from *Micrococcus luteus* B-P 26 is composed of two dissociable protein components, A and B (*4*, *5*). Similarly, two essential components, I and II, constituting heptaprenyl diphosphate (HepPP, C_{35}) synthase were separated and characterized from *Bacillus subtilis* (*6*, *7*). These medium-chain (*E*)-prenyl diphosphate synthases have novel heterodimeric structures with subunits easily dissociable under physiological conditions. The genes encoding the two enzymes have been cloned and sequenced (*8*, *9*). The putative amino acid sequences show that the larger subunits (components B and II, or Hexs-B and GerC3, respectively) have conserved regions that are characteristic of (*E*)-prenyltransferases, whereas the smaller subunits (components A and I, or Hexs-A and GerC1, respectively) have little similarity to any protein sequence of the other classes of prenyltransferases. The two dissociable subunits of each of these medium-chain (*E*)-prenyl diphosphate synthases have been shown to associate with each other in the presence of allylic substrate FPP and Mg^{2+} to form a

† This work was supported in part by Grant-in-Aid for Encouragement of Young Scientist (11780412 to Y.-W.Z.) from the Ministry of Education, Science, and Culture, Japan, by the Research for the Future Program (JSPS-RFTF97I00302 to T.K.) from the Japan Society for the Promotion of Science, and by the Asahi Glass Foundation.

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[1] Abbreviations: BSA, bovine serum albumin; DMAPP, dimethylallyl diphosphate; FARM, first aspartate-rich motif; FGPP, farnesylgeranyl diphosphate; FPP, farnesyl diphosphate; GPP, geranyl diphosphate; GGPP, geranylgeranyl 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.

10.1021/bi001311p CCC: $19.00
Published on Web 09/22/2000

catalytically active complex, which represents an intermediary state during catalysis (*4*, *10*). Recently, site-directed mutagenetic studies of the smaller subunit (component I) of *B. subtilis* HepPP synthase have revealed that several amino acid residues in region B are involved in the binding of allylic substrate (*11*).

Ohnuma et al. (*12*−*16*) have intensively investigated the chain length determination mechanism of short-chain prenyl diphosphate synthases such as *Bacillus stearothermophilus* FPP synthase and *Sulfolobus acidocaldarius* geranylgeranyl diphosphate (GGPP) synthase. In their mutational analyses, the chain length of the final product of these enzymes was dramatically changed by replacement of only one aromatic amino acid, situated at the fifth amino acid upstream to the first aspartate-rich motif (FARM). They suggested that this bulky residue directly blocks the condensation reaction and provides the product specificities of these bacterial enzymes (*13*). Furthermore, by comparison with eukaryotic short-chain prenyl diphosphate synthases, they proposed that the region from the fifth position upstream to the FARM to the end of the FARM sequence precisely determines the chain lengths of the final products of all short-chain prenyl diphosphate synthases (*16*). On the other hand, Tarshis et al. (*17*) have reported that the two aromatic residues, located at the fourth and fifth positions upstream to the FARM in avain FPP synthase, form the floor of the putative allylic substrate binding pocket and are important for controlling product chain length. These observations indicate that simple combinations of characteristic amino acid residues in the chain length determination region confer product specificities upon prenyltransferases.

The larger subunits of medium-chain (*E*)-prenyl diphosphate synthases have an amino acid residue, Ala, at the fifth position upstream to the FARM, which is smaller and more flexible compared with those of short-chain prenyl diphosphate synthases. Recently, the two conserved amino acid residues in the smaller subunits of medium-chain (*E*)-prenyl diphospahte synthases have been shown to be involved in determining the chain length of reaction products (*11*). As each of these enzymes essentially requires the coexistence of both subunits for its catalysis, this may raise the possibility that this group of prenyltransferases takes a novel mode for the product chain length determination, in which both subunits are included. To better understand the prenyl chain length determination mechanism of medium-chain (*E*)-prenyl diphosphate synthases, we selected several amino acid residues in the larger subunits of *M. luteus* HexPP synthase and *B. subtilis* HepPP synthase for substitutions by site-directed mutagenesis and examined their effects on the prenyl chain length of reaction products by combination with the corresponding wild-type or mutated smaller subunits. This paper describes the results, demonstrating that both subunits are responsible for the chain length determination of medium-chain (*E*)-prenyl diphosphate synthases.

EXPERIMENTAL PROCEDURES

Materials and General Procedures. [1-^{14}C]IPP (1.95 TBq/mol) was purchased from Amersham. Nonlabeled IPP, dimethylallyl diphosphate (DMAPP), geranyl diphosphate (GPP), FPP, and GGPP were synthesized according to the procedure of Davisson et al. (*18*). Restriction enzymes and other DNA-modifying enzymes were from Takara Shuzo Co., Ltd., and Toyobo Co., Ltd. *Escherichia coli* K12 strain TOP10 (Invitrogen) 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. (*19*). Bacteria were cultured in SOB or M9YG medium (*20*). The wild-type components of *M. luteus* B-P 26 HexPP synthase and *B. subtilis* HepPP synthase were overproduced in *E. coli* cells and purified separately essentially according to the procedure previously described (*10*). All other chemicals were of analytical grade.

Site-Directed Mutagenesis. To introduce mutations in the structural gene, *hexs-b* for the component B protein of *M. luteus* HexPP synthase, a PCR-amplified fragment including the *hexs-b* gene from the clone pHX06 (*9*) was subcloned into pUC119 (Takara Shuzo), yielding pUHexsB. Similarly, the fragment containing *gerC3* for the component II protein of *B. subtilis* HepPP synthase from pSE01 (*10*) was ligated into pUC119, yielding pUGerC3. Site-directed mutagenesis was conducted with the double-stranded DNA template of pUHexsB or pUGerC3 according to the protocol of the GeneEditor in vitro site-directed mutagenesis system (Promega). The mutagenic oligonucleotides designed to produce the desired point mutations were as follows: Hexs-B/V76G, 5′-ACTTGCCATATGACCGAGCTCCAGAG-3′; Hexs-B/A79L, 5′-CTCGTTCATATGCTAAGTCTCGTTCATG-3′; Hexs-B/A79F, 5′-CTCGTTCATATGTTCAGTCTCGTTCATG-3′; GerC3/I76G, 5′-ATGCCATGTGACCCATTTCCAGAGTG-3′; GerC3/A79L, 5′-GATTCACATGCTATCTTTGGTTCATG-3′; GerC3/A79F, 5′-GATTCACATGTTCTCTTTGGTTCATG-3′ (mismatched bases are underlined). Introduction of the mutation was confirmed by sequencing the whole nucleotide sequences using the dideoxy chain termination method (*21*) with a DNA sequencer (LI-COR, model 4200).

Construction of Expression Vector Systems for Wild-Type and Mutated Larger Subunits. An expression vector system for Hexs-B or GerC3 was constructed as N-terminal His-tagged fusion proteins suitable for rapid purification (pTrcHis system, Invitrogen). In both cases, full-length genes were amplified by using the appropriate sense and antisense primers, which were used for introduction of a *Bam*HI site and an *Eco*RI site downstream to the stop codon, respectively. The PCR products were digested with *Bam*HI and *Eco*RI, and the resulting fragments were ligated into pTrcHis A (for *hexs-b*) or pTrcHis B (for *gerC3*) vector digested with the same restriction enzymes, yielding the wild-type expression plasmid, pHisHexsB or pHisGerC3, respectively. Each of the mutated plasmids (see above) was digested with *Bsi*WI and *Eco*RI and then separated on an agarose gel. The desired band was eluted and ligated into the corresponding pHisHexsB or pHisGerC3, respectively, which was digested with *Bsi*WI and *Eco*RI, to construct each of the mutation expression plasmids.

Overproduction and Purification of Wild-Type and Mutated Larger Subunits. Each of the expression plasmids was used for transformation of *E. coli* TOP10, and 100 μL of the overnight culture of the transformant in LB medium containing 50 μg/mL ampicillin was inoculated into 50 mL of SOB medium containing 50 μg/mL ampicillin. The cells were grown at 37 °C to an approximate A_{600} value of 0.5, isopropyl β-D-thiogalactopyranoside (IPTG) was added to a

Table 1: Kinetic Constants of the Mutated Enzymes[a]

	GPP		FPP		GGPP		IPP[b]
enzyme	K_m (μM)	V_{max}[c] (nmol min^{-1} nmol^{-1})	K_m (μM)	V_{max}[c] (nmol min^{-1} nmol^{-1})	K_m (μM)	V_{max}[c] (nmol min^{-1} nmol^{-1})	K_m (μM)
A-WT/B-WT	56.1 ± 8.9	265 ± 60	8.9 ± 2.4	1420 ± 120	12.4 ± 3.2	920 ± 90	18.4 ± 3.5
A-WT/B-A79L	9.5 ± 2.1	1830 ± 110	7.5 ± 2.4	1657 ± 134	47.8 ± 12.2	385 ± 65	21.6 ± 5.8
A-WT/B-A79F	5.6 ± 1.4	2846 ± 255	6.7 ± 1.9	2120 ± 188	61.4 ± 21.5	342 ± 83	18.6 ± 4.1
A-WT/B-V76G	46.3 ± 10.3	176 ± 44	10.5 ± 3.4	1564 ± 108	17.6 ± 4.1	804 ± 44	13.7 ± 2.4
I-WT/II-WT	66.2 ± 7.5	255 ± 38	7.7 ± 1.8	1252 ± 100	16.8 ± 4.7	856 ± 59	19.8 ± 4.6
I-WT/II-A79L	77.8 ± 9.0	301 ± 50	12.4 ± 2.6	980 ± 106	34.4 ± 12.5	280 ± 20	25.9 ± 6.7
I-WT/II-A79F	73.4 ± 8.3	372 ± 72	8.5 ± 2.6	1540 ± 189	56.8 ± 18.7	410 ± 35	14.3 ± 3.2
I-WT/II-I76G	69.4 ± 12.6	288 ± 64	11.6 ± 2.8	874 ± 80	19.8 ± 3.9	748 ± 69	21.4 ± 5.2

[a] The combination of the wild-type smaller subunit and an equimolar wild-type or mutated larger subunit was used as the heteromeric enzyme for kinetic studies. [b]For the reaction with FPP. [c]Nanomoles of IPP converted to products per minute by 1 nmol of enzyme.

final concentration of 1 mM, and then the incubation was continued for additional 3 h at 30 °C. Overproduction of the proteins was confirmed by sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE).

Each of the His-tagged proteins overproduced in *E. coli* cells was purified essentially according to the protocol of the Xpress protein purification system (Invitrogen), using a Ni^{2+} resin column with elution of a gradient of 0−500 mM imidazole in 20 mM phosphate buffer, pH 6.0. A portion of the purified fusion protein was treated with enterokinase (Novagen) to remove the N-terminal fusion peptide, and the resulting protein was used for enzymatic activity assay and product analysis. Protein concentrations were measured by the method of Bradford (*22*) with bovine serum albumin (BSA) as a standard.

Enzymatic Activity Assay and Product Analysis. The enzymatic activity was measured by determination of the amounts of [1-^{14}C]IPP incorporated into butanol-extractable polyprenyl diphosphates. A standard assay mixture contained, in a final volume of 0.3 mL, 15 μM [1-^{14}C]IPP (37 MBq/mol), 25 mM Tris-HCl buffer, pH 8.5, 25 mM NH_4Cl, 10 mM 2-mercaptoethanol, 1 mM $MgCl_2$, 15 μM allylic substrate, and a suitable amount of enzyme solution. After incubation at 37 °C for 30 min, the reaction products were 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 (*23*) and analyzed with 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. Relative amounts of products were calculated from the intensities of the photostimulated luminescence on the imaging plate.

RESULTS

Production of Site-Directed Mutants of Larger Subunits of Medium-Chain (E)-Prenyl Diphosphate Synthases. A comparison of the deduced amino acid sequences of (*E*)-prenyltransferases around the conserved FARM shows a common profile. Short-chain prenyl diphosphate synthases such as FPP synthases and archaeal GGPP synthases have an aromatic amino acid, Phe or Tyr, at the position of five residues before the FARM. In contrast, other GGPP synthases and some higher chain length synthases have an aliphatic amino acid residue, Ala, Ser, or Met, at the corresponding position. Substitution of the aromatic residue at this position with an amino acid having a smaller side chain resulted in the conversion of *B. stearothermophilus* FPP synthase to GGPP synthase or *S. acidocaldarius* GGPP synthase to the enzyme catalyzing the synthesis of C_{30-50} diphosphates (*13*, *14*). Replacements of *B. stearothermophilus* FPP synthase with other amino acids also brought about different chain lengths of the final products, which are inversely proportional to the accessible surface area of the substituted amino acid (*15*). Double mutation of *B. stearothermophilus* FPP synthase at both the fifth and the eighth positions before the FARM with Gly yielded a final product with a C_{35} prenyl chain length (*12*).

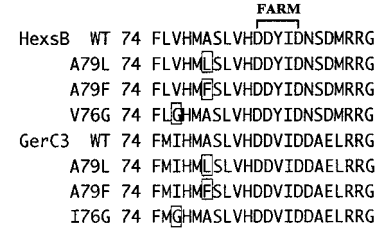

FIGURE 1: Amino acid sequences around the FARM of the wild-type and mutated HexPP synthases of *M. luteus* B-P 26 and HepPP synthases of *B. subtilis*. The mutated amino acid residues were boxed.

On the other hand, medium-chain (*E*)-prenyl diphosphate synthases catalyze similar condensation reactions, and the larger subunits of these enzymes have sequence similarities to short-chain prenyl diphosphate synthases. On the basis of this information, we changed the Ala at the fifth position and Val or Ile at the eighth position upstream to the FARM in *M. luteus* HexPP synthase or *B. subtilis* HepPP synthase, respectively, to construct mutant subunits of Hexs-B, B-A79L, B-A79F, and B-V76G, respectively, or of GerC3, II-A79L, II-A79F, and II-I76G, respectively (Figure 1).

Kinetic Analysis of the Mutated Enzymes. The mutated larger subunits were combined with the corresponding wild-type counterpart smaller subunits, A-WT or I-WT, and kinetic constants of these mutated enzymes were determined by employing GPP, FPP, or GGPP as allylic substrates (Table 1). All mutated enzymes showed Michaelis constants (K_m) for the homoallylic substrate IPP comparable to that of the wild-type, suggesting that these substitutions have little effect

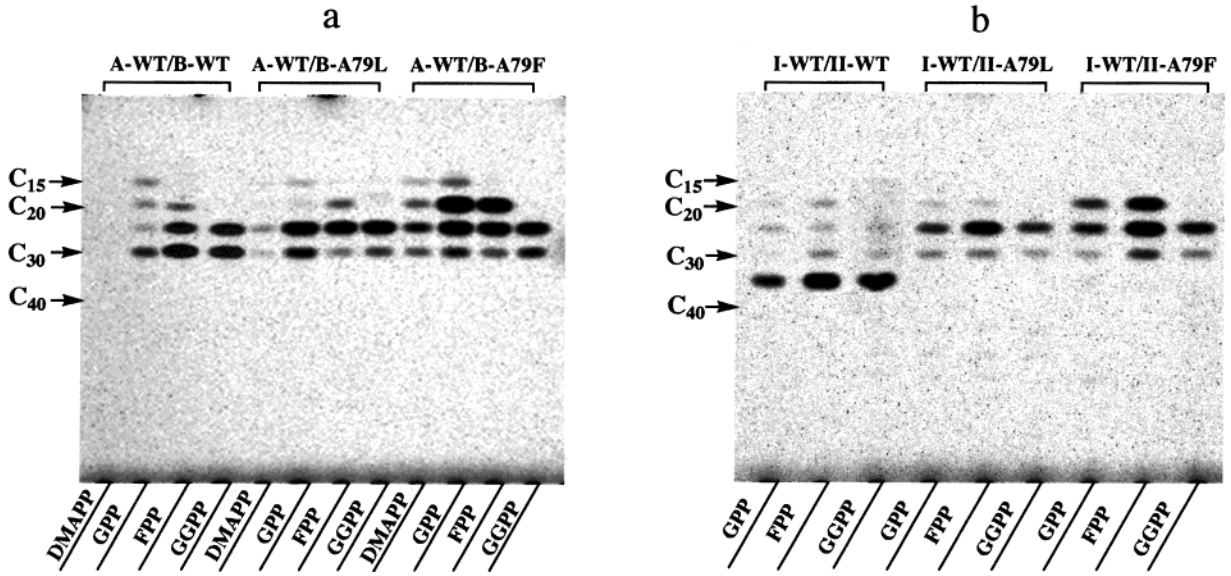

FIGURE 2: Product analyses of the reaction catalyzed by the wild-type or 79-mutated HexPP synthases (a) and HepPP synthases (b). The alcohols obtained by enzymatic hydrolysis of the products formed by the incubation of [1-^{14}C]IPP and DMAPP, GPP, FPP, or GGPP with the wild-type or 79-mutated enzymes were analyzed by reversed-phase TLC as described under Experimental Procedures. The HexPP synthase mutants (a) or HepPP synthase mutants (b) and the allylic substrates used in each of the enzymatic reactions are indicated at the top and the bottom of each lane of the TLC autoradiograms, respecitively. The TLC plates were 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_{30}, (*all-E*)-hexaprenol; C_{45}, (*all-E*)-nonaprenol.

on the binding of IPP. When the assay was carried out using GPP or FPP as an allylic primer, the mutated HepPP synthases having replacements at the 79 position showed comparable kinetic constants with those of the wild-type enzyme. On the other hand, the mutated HexPP synthases having replacements at the same position showed 7−10-fold increased V_{max} values and 5−10-fold decreased K_m values for GPP, which became the most preferred substrate of the allylic primers. Although these substitutions at the 79 position resulted in 2−5-fold increased K_m values for GGPP, all of the 79-mutated enzymes showed moderate levels of enzymatic activity. However, no significant difference in kinetic constants was observed between the mutated enzymes having replacements at the 76 position and the wild type.

Product Analysis of the Mutated Enzymes. The chain length distributions and their amounts of the prenyl alcohols derived from the enzymatic reaction products were analyzed by reversed-phase TLC. Both 79-mutated HexPP synthases could synthesize HexPP as its final products with any of the four allylic primers (Figure 2a). However, the product distributions of these mutated enzymes are quite different from that of the wild-type HexPP synthase. Replacement of Ala-79 with Leu or Phe resulted in shortening the chain length of the major product. A-WT/B-A79L produced farnesylgeranyl diphosphate (FGPP, C_{25}) as its major product, whereas GGPP and FGPP became major by the reaction of A-WT/B-A79F.

On the other hand, 79-mutated HepPP synthases showed a significantly different pattern of product distribution from that of the wild type (Figure 2b). Neither I-WT/II-A79L nor I-WT/II-A79F could produce HepPP as its final products with any of the allylic primers. As shown in Figure 2b, these mutant enzymes produced HexPP as the final product. I-WT/II-A79L gave FGPP as the major product, whereas I-WT/II-A79F yielded a mixture of GGPP and FGPP as the major product.

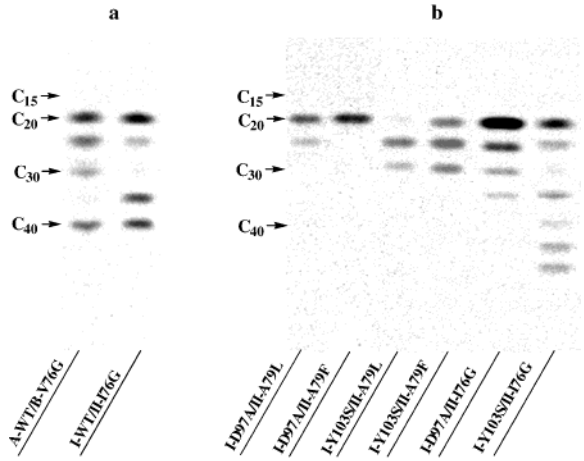

FIGURE 3: TLC autoradiograms of the alcohols obtained by enzymatic hydrolysis of the products formed by the incubation of [1-^{14}C]IPP and FPP with 76-mutated HexPP synthase A-WT/B-V76G and 76-mutated HepPP synthase I-WT/II-I76G (a) or the double-mutated HepPP synthases (b). The mutants of HexPP synthase or HepPP synthase used in each of the enzymatic reactions are indicated at the bottom of each lane of the TLC autoradiograms. Arrowheads show the positions of authentic alcohols as described in the legend to Figure 2.

Both 76-mutated HexPP synthase (A-WT/B-V76G) and 76-mutated HepPP synthase (I-WT/II-I76G) gave octaprenyl diphosphate (C_{40}) as the final products with FPP as an allylic primer (Figure 3a), suggesting that the residue at the eighth position before the FARM is involved in determining prenyl chain elongation in these wild-type medium-chain (*E*)-prenyl diphosphate synthases. However, these mutants showed increases in production of intermediate products compared with the wild-type enzymes. 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 (*15*). Presumably, the mutations cause ad-

ditional structural changes that affect the affinity of the enzyme for reaction intermediates.

Reaction Catalyzed by Double-Mutated HepPP Synthases. Recently, we have reported that the two mutants (I-D97A and I-Y103S) with respect to region B of component I (GerC1) of *B. subtilis* HepPP synthase show significant changes in chain length distribution from the wild-type enzyme (*11*). Combination of I-D97A with wild-type component II, I-D97A/II-WT, was shown to produce GGPP as a main product as well as FGPP, HexPP, and HepPP, while combination of the other mutant, I-Y103S, with wild-type component II, I-Y103S/II-WT, gave a final product with C_{40} prenyl chain length. To examine the effects of assembly of the two heteromeric mutant subunits, both of which were found to give changes in the prenyl chain length of the reaction product, I-D97A or I-Y103S was combined with II-A79L, II-A79F, or II-I76G, respectively, to form six combinations of double-mutated HepPP synthases, I-D97A/II-A79L, I-D97A/II-A79F, I-D97A/II-I76G, I-Y103S/II-A79L, I-Y103S/II-A79F, and I-Y103S/II-I76G. The specific activities of these double-mutated enzymes were determined using GPP, FPP, or GGPP as an allylic primer. When the assay was carried out using GPP or FPP, all mutated enzymes showed comparable levels of prenyltransferase activities with that of combination of the wild-type subunits. On the other hand, enzyme activities for GGPP are different. The activities of wild-type enzyme and I-Y103S/II-I76G were 2−3-fold higher than those of I-Y103S/II-A79L, I-Y103S/II-A79F, and I-D97A/II-I76G, whereas no significant prenyltransferase activity was observed with I-D97A/II-A79L or I-D97A/II-A79F. The enzymatic reaction products of these double-mutated enzymes with FPP and [1-^{14}C]IPP as substrates were analyzed by TLC. To our surprise, significant changes in the product distribution spectra of the mutated enzymes were observed. As shown in Figure 3b, I-D97A/II-A79L gave FGPP as the final product and an increase in the production of GGPP, while I-D97A/II-A79F formed GGPP exclusively. I-Y103S/II-A79L and I-Y103S/II-A79F showed a product distribution pattern similar to those by I-WT/II-A79L or I-WT/II-A79F. On the other hand, I-D97A/II-I76G formed prenyl diphosphates with a product distribution pattern similar to that of I-D97A/II-WT but different from that of I-WT/II-I76G. When compared with I-Y103S/II-WT and I-WT/II-I76G, both of which could form C_{40} prenyl diphosphate, I-Y103S/II-I76G acquired further ability to synthesize longer prenyl chains up to decaprenyl diphosphate (C_{50}).

DISCUSSION

Comparison of the primary structures of (*E*)-prenyltransferases revealed a commom product chain length determination region around the FARM (*2*). The aromatic amino acid residue located at the fifth position upstream to the FARM in short-chain prenyl diphosphate synthases was shown to form the floor of the allylic substrate binding pocket and provide the distinct product specificity (*17*). In contrast, higher chain length synthases have an amino acid residue with a smaller and more flexible side chain at this position, conferring the catalytic functions to produce longer prenyl chain products on these enzymes. All of the larger subunits of medium-chain (*E*)-prenyl diphosphate synthases, whose sequences have been revealed so far, have a common amino acid residue, Ala, at this position. Replacement of this Ala in the larger subunits of *M. luteus* HexPP synthase and *B. subtilis* HepPP synthase with the bulky amino acids resulted in shortening the chain length of final or/and major products of these enzymes. These results support the view that the amino acid residue at the fifth position upstream to the FARM plays an important role in determining the product chain length of all (*E*)-prenyl diphosphate synthases (*2*).

The conserved region B in smaller subunits of the medium-chain (*E*)-prenyl diphosphate synthases was shown to be involved in the binding of allylic substrate as well as the chain length determination (*11*). This may raise the possibility that these enzymes have a novel mechanism for product chain length determination, in which not only the larger subunit but also the smaller one are included. It is of particular interest to learn the cooperative action of both subunits on product chain length determination. Replacement of the charged residue Asp at the position of 97 with an aliphatic amino acid, Ala, in region B of the smaller subunit of *B. subtilis* HepPP synthase led to the formation of GGPP as its main product with a considerable amount of longer chain prenyl diphosphates (*11*). The mutated HepPP synthase I-WT/II-A79L produced FGPP as its major product as well as a minor amount of GGPP and HexPP, while the combination of mutated components, I-D97A/II-A79L, resulted in a markedly different product distribution spectrum, in which GGPP is its main product with a minor amount of FGPP. Moreover, the I-D97A/II-A79F enzyme formed GGPP exclusively. These results indicate that the two amino acid residues, Asp-97 in the smaller subunit and Ala-79 in the larger one, cooperatively participate in maintaining the product specificity of *B. subtilis* HepPP synthase. Another mutant, I-Y103S/II-WT, of *B. subtilis* HepPP synthase was shown to produce a final product of C_{40} prenyl chain length (*11*). The combination of the I-Y103S mutated smaller subunit and the mutated larger one, II-A79L or II-A79F, I-Y103S/II-A79L or I-Y103S/II-A79F, gave a product distribution profile similar to that of the combination of wild-type component I and II-A79L or II-A79F, I-WT/II-A79L or I-WT/II-A79F. Therefore, it is reasonable to assume that the Tyr-103 of component I resides in a farther remote position than the Asp-97 of the same component from the FARM of component II in the heterodimeric structure and plays an important role in determining product specificity of this enzyme. A hydrophobic residue, Val (Hexs-B) or Ile (GerC3), at the eighth position before the FARM was shown to block the growing prenyl chain in the wild type. However, this Ile residue is also conserved at the corresponding position in several enzymes that catalyze the formation of prenyl diphosphates with chain lengths longer than C_{35}, including octaprenyl diphosphate synthases from *E. coli* (*24*) and *Treponema pallidum* (*25*) and decaprenyl diphosphate synthases from *Schizosaccharomyces pombe* (*26*) and *Gluconobacter suboxydans* (*27*). The solanesyl diphosphate synthase from *Rhodobacter capsulatus* has a Leu residue at the corresponding position (*28*). Determination of product specificity in *B. subtilis* HepPP synthase may involve the cooperative action of Tyr-103 of component I and Ile-76 of component II, because the double-mutated enzyme I-Y103S/II-I76G yielded a longer prenyl chain product than that formed by either I-Y103/II-WT or I-WT/II-I76G. Taken together, these results give several hypothetical models for determining product chain length in *B. subtilis* HepPP

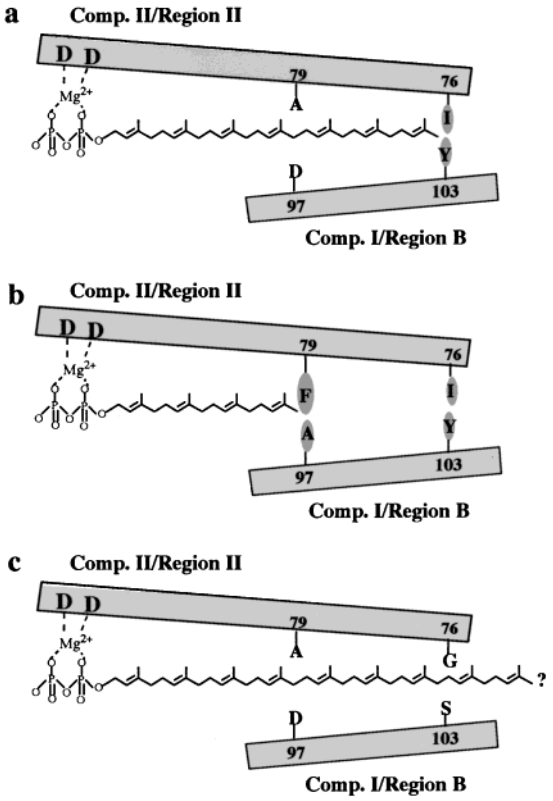

FIGURE 4: Hypothetical models for the product chain length determination between the two subunits of *B. subtilis* HepPP synthase: (a) wild-type HepPP synthase; (b) I-D97A/II-A79F mutant; (c) I-Y103S/II-I76G mutant. Abbreviations: Comp. I, component I; Comp. II, component II.

synthase, in which both subunits are included (Figure 4). The two amino acid residues, Asp-97 in component I and Ala-79 in component II, are responsible for proceeding the chain elongation beyond GGPP (Figure 4b), and the other two residues from different subunits, Tyr-103 in component I and Ile-76 in component II, are involved in determining the prenyl chain length of the products of this enzyme (Figure 4c).

The two 79-mutated HexPP synthases, A-WT/B-A79L and A-WT/B-A79F, showed dramatic increases in accepting the short-chain prenyl diphosphate GPP as an allylic substrate compared with the wild type (Table 1). It seems that replacement of the Ala at the fifth position before the FARM with bulky amino acids led to reduction of the size of the hydrophobic pocket for binding of allylic substrate and some movement of the protein side chain as well, so that the 79-mutated HexPP synthases, A-WT/B-A79L and A-WT/B-A79F, can accept GPP as a preferred substrate like short-chain prenyl diphosphate synthases such as FPP synthase and GGPP synthase. In contrast, similar substitutions in HepPP synthase gave no significant difference in accepting the short-chain prenyl diphosphates from the wild-type enzyme. In addition, though several mutated HepPP synthases could give HexPP as their final products, no combination between the two subunits from the two different enzymes showed a prenyltransferase activity (data not shown). Hence, it is reasonable to assume that there is a significant structural difference between the HexPP synthase and the HepPP synthase, especially in the hydrophobic pocket for binding of allylic substrate and growing prenyl chain. Determination of the crystal structures of these heteromeric medium-chain (*E*)-prenyl diphosphate synthases may accelerate the understanding of the molecular mechanism of these unusual prenyl chain elongating enzymes.

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BI001311P