Two Subunits of Heptaprenyl Diphosphate Synthase of *Bacillus subtilis* Form a Catalytically Active Complex†

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ABSTRACT: Heptaprenyl diphosphate synthase of *Bacillus subtilis*, which participates in the biosynthesis of the side chain of menaquinone-7, is composed of two dissociable subunits, component I and component II, which are encoded by two cistrons in a novel gene cluster of *gerC* operon [Zhang, Y.-W., et al. (1997) *J. Bacteriol. 179*, 1417−1419]. This enzyme essentially requires the coexistence of both subunits for its catalysis. Expression vector systems for the two structural genes, *gerC1* and *gerC3*, were constructed separately, and the two components were overproduced in *Escherichia coli* cells. After purification, their dynamic interactions in forming a catalytically active complex were investigated by gel filtration and immunoblotting analyses. When a mixture of the two components that had been preincubated in the presence of Mg^{2+} and farnesyl diphosphate was subjected to Superdex 200 gel filtration, a significant elution peak appeared in a region earlier than those observed when they were chromatographed individually. This fraction contained both components I and II, and it corresponded to a molecular mass that is in accord with the sum of the values of the two components. Cross-linking studies indicate that the two essential subunits, farnesyl diphosphate, and Mg^{2+} form a ternary complex which seems to represent a catalytically active state of the heptaprenyl diphosphate synthase. On the other hand, no complex was formed in the presence of isopentenyl diphosphate or inorganic pyrophosphate and Mg^{2+}. A photoaffinity analogue of farnesyl diphosphate was shown to preferentially label the component I protein, suggesting that component I possesses a specific affinity for the allylic substrate. Furthermore, the photoaffinity labeling of component I significantly increased in the presence of component II. The mechanism of catalysis of this unique heteromeric enzyme is understood by assuming that association and dissociation of the two subunits facilitate turnover of catalysis for the synthesis of the amphipathic product from soluble substrates.

Prenyltransferases are the enzymes that catalyze the sequential condensation of isopentenyl diphosphate (IPP)[1] with allylic diphosphates to give prenyl diphosphates in the biosynthetic pathway of isoprenoid compounds. Although these condensation reactions are similar in terms of chemical mechanism, there are a number of enzymes having different specificities with respect to the chain length and double-bond stereochemistry of substrates and products. These enzymes are extremely interesting from an enzymological viewpoint in that the reactions are regulated to proceed consecutively and terminate precisely at definite chain lengths depending on their specificities.

So far, a number of prenyltransferases have been found from bacteria and characterized. These enzymes can be classified into four groups according to the modes of requirement for enzymatic activity as well as the chain length and stereochemistry of reaction products (*1*). Farnesyl diphosphate (FPP) synthase and geranylgeranyl diphosphate (GGPP) synthase require only divalent metal ions (e.g., Mg^{2+} or Mn^{2+}) for their activities; these ions are generally required by all prenyl diphosphate synthases. Octaprenyl diphosphate synthase, solanesyl diphosphate (SPP) synthase, and decaprenyl diphosphate synthase, which catalyze the formation of long-chain (*E*)-prenyl diphosphates, require additional protein factors that may be involved in removing the hydrophobic polyprenyl products from their active sites to facilitate and maintain the turnover of catalysis (*2*). Prenyltransferases catalyzing the synthesis of (*Z*)-polyprenyl diphosphates require phospholipid or detergent (*3*). All of these prenyl diphosphate synthases have tightly coupled homodimeric structures. On the other hand, heptaprenyl

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[1] Abbreviations: IPP, isopentenyl diphosphate; DMAPP, dimethylallyl diphosphate; GPP, geranyl diphosphate; FPP, farnesyl diphosphate; GGPP, geranylgeranyl diphosphate; HexPP, hexaprenyl diphosphate; HepPP, heptaprenyl diphosphate; SPP, solanesyl diphosphate; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; AP, alkaline phosphatase; IPTG, isopropyl β-D-thiogalactopyranoside; Tris, tris(hydroxymethyl)aminomethane; DIG, digoxigenin; PCR, polymerase chain reaction; TLC, thin layer chromatography; DSS, disuccinimidyl suberate; *m*-Bz-C_{10}-OPP, (8-*m*-benzoylphenoxy)geranyl diphosphate; PP_i, inorganic pyrophosphate.

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diphosphate (HepPP) synthase from *Bacillus subtilis* (*4*−*6*) and *Bacillus stearothermophilus* (*7*) and hexaprenyl diphosphate (HexPP) synthase from *Micrococcus luteus* B-P 26 (*8*−*10*), which catalyze the formation of medium-chain (*E*)-prenyl diphosphates, belong to an exceptional group of the enzymes because they comprise two nonidentical protein components, neither of which has catalytic activity alone.

During the past decade, the structural genes or cDNAs for many prenyltransferases have been identified and characterized (*11*). Comparison of the primary structures of the deduced amino acid sequences of these enzymes revealed the presence of seven highly conserved regions typical of prenyltransferases (*12*). On the basis of the highly conserved amino acid sequences, we have identified the genes encoding HepPP synthase of *B. subtilis* (*6*) and *B. stearothermophilus* (*7*) and HexPP synthase of *M. luteus* B-P 26 (*13*), confirming that each of these medium-chain (*E*)-prenyl diphosphate synthases comprises two essential protein components. The deduced amino acid sequences show that one of the two subunits of each of the three enzymes has highly conserved regions that are characteristic of (*E*)-prenyl diphosphate synthases, including two aspartate-rich regions, while the other has no similarity to the protein sequences registered so far in protein databases (*7*). It has been proposed that one of the two subunits supplies substantial sites for substrate binding and catalysis, whereas the other plays an auxiliary but essential role in catalytic function (*6*). Hence, it is particularly interesting to determine why these medium-chain (*E*)-prenyl diphosphate synthases must take two-component systems in contrast to the other classes of prenyl diphosphate synthases having tightly coupled homodimeric structures.

HepPP synthase of *B. subtilis* is composed of two dissociable subunits with molecular masses of 29 (component I) and 36 kDa (component II), which are encoded by two cistrons in a gene cluster of the *gerC* operon (*6*). To explore the dynamic interaction of the two dissociable components during catalysis, expression vector systems for the two structural genes, *gerC1* and *gerC3*, were constructed separately, and the two components were overproduced in *Escherichia coli* cells. Then these components were purified, and their behavior was investigated. This paper describes the results demonstrating a catalytically active complex formed between the two subunits of this medium-chain (*E*)-prenyl diphosphate synthase.

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 (*E*,*E*)-FPP were synthesized according to the procedure of Davisson et al. (*14*). [1-^{3}H]-(8-*m*-Benzoylphenoxy)geranyl diphosphate (*m*-Bz-C_{10}-OPP, 129.5 MBq/mmol) was the same preparation described previously (*15*). Lysozyme, ribonuclease, and acid phosphatase were products of Sigma. Precoated reversed phase thin layer chromatography (TLC) plates, LKC-18, were purchased from Whatman. 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. Chromosomal DNA of *B. subtilis* ISW1214 was isolated according to the method described by Saito and Miura (*16*). Restriction enzyme digestions, transformations, and other standard molecular biology techniques were carried out as described by Sambrook et al. (*17*). Bacteria were cultured in Luria-Burtani (LB) or M9YG medium (*12*). Alkaline phosphatase (AP)-conjugated goat anti-mouse IgG was a product of ICN Pharmaceuticals Inc. All other chemicals were of analytical grade.

Construction of a B. subtilis Genomic Library. The chromosomal DNA was partially digested with *Fba*I and developed on a 0.8% agarose gel. The DNA fragments that were approximately 6 kbp long were extracted from the gel matrix by glassmilk (Bio 101, Inc.) and inserted into the *Bam*HI site of pUC 119 by T4 DNA ligase, and then the ligation mixture was used directly for transformation of *E. coli* JM109 cells to construct a genomic library of *B. subtilis*.

Southern Blotting Analysis. The *gerC1* or *gerC3* gene region amplified by polymerase chain reaction (PCR) (*6*) was labeled enzymatically with digoxigenin (DIG)-dUTP as a DNA hybridization probe. The partial digest of the genomic DNA was electrophoresed, blotted, and hybridized with the labeled probes. The hybridized probes were immunodetected with sheep anti-DIG, Fab fragment AP conjugate according to the protocol of the DIG DNA detection kit (Boehringer Mannheim).

Colony Hybridization. Approximately 5000 transformants were screened for the genomic fragment hybridizing to the labeled probes (see above). Positive colonies were cultured in LB medium and examined for the expression of HepPP synthase activity using [1-^{14}C]IPP and FPP as substrates.

Construction of the Expression Vector Systems for HepPP Synthase. A 2.9 kbp DNA fragment containing the *gerC* gene region of *B. subtilis* was excised from a plasmid pHEP02, which was isolated from one of the positive clones producing HepPP synthase, with *Xba*I. The fragment was ligated into the *Xba*I site of pUC 119 to give pHEP04, which was then digested with *Stu*I−*Hin*cII or *Stu*I−*Eco*RI, and the resulting fragment was inserted separately into the *Sma*I or *Sma*I−*Eco*RI sites of pUC 119 to subclone *gerC1* or *gerC3* and obtain plasmids pSH01 and pSE01, respectively (Figure 1).

To construct expression plasmids for the two components, we introduced several mutations in pSH01 or pSE01 by the method of Kunkel et al. (*18*). Two pairs of mutagenic oligonucleotide primers, 5′-AAGGGTGATAT**GCATGC**AAGACAT-3′ and 5′-AAGGGTAAACCATT**CTGCAG**GACTC-3′, and 5′-TGGGTGAATG**TCATGA**AATTTAAAATGGCC-3′ and 5′-TGTAGATAT**TCTAGA**AATTTCTCCGCCCCGG-3′ (mismatched bases are underlined, newly created restriction sites are bold), were designed to create an *Sph*I (pSH01) or *Bsp*HI (pSE01) site immediately upstream of the predicted translation initiation codons, TTG of *gerC1* or ATG of *gerC3*, respectively, and a *Pst*I or *Xba*I site at seven (*gerC1*) or 14 bases (*gerC3*) downstream of the termination codon, TAA, respectively. Introduction of these restriction sites was confirmed by the dideoxy chain termination method (*19*) with a DNA sequencer (LI-COR, model 4200). Then, the *Sph*I−*Pst*I fragment of pSH01 containing the *gerC1* region, was inserted into *Nco*I−*Pst*I sites of pTrc99A (Pharmacia Biotech Inc.) to construct a *gerC1* expression plasmid, pHAEX01. Similarly, the *Bsp*HI−*Xba*I fragment of pSE01 was inserted into the *Nco*I−*Xba*I sites of pTrc99A to give a *gerC3* expression plasmid, pHAEX03 (Figure 1).

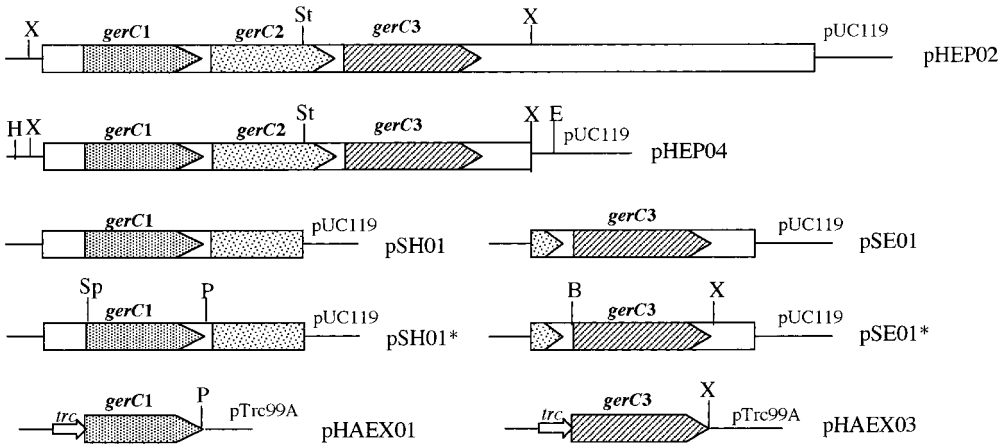

FIGURE 1: Schematic presentation of expression vector systems for HepPP synthase of *B. subtilis*. Two plasmids, pSH01* and pSE01*, were derived from pSH01 and pSE01 by site-directed mutagenesis, respectively, having new restriction sites as described in Experimental Procedures. Restriction sites are as follows: X, *Xba*I; H, *Hin*cII; St, *Stu*I; E, *Eco*RI; P, *Pst*I; B, *Bsp*HI; and Sp, *Sph*I.

Overproduction of GerC1 (Component I) or GerC3 (Component II). After incubation in LB medium containing 50 μg/mL ampicillin at 37 °C overnight, each of the transformants, pHAEX01/JM109 or pHAEX03/JM109, was inoculated into a 250-fold volume of M9YG medium containing 50 μg/mL ampicillin. The cells were grown at 37 °C to an A_{600} value of approximately 0.6; isopropyl β-D-thiogalactopyranoside (IPTG) was added to a 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).

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. The assay mixture contained, in a final 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 30 °C for 30 min, the reaction products were immediately extracted with 1-butanol saturated with water. 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 (*20*), and analyzed by reversed phase TLC with a solvent system of acetone/water (19/1).

During purification of each of the components, the HepPP synthase activity was measured by supplying the corresponding counterpart component.

Purification of the Two Essential Components. E. coli JM109 cells harboring pHAEX01 or pHAEX03 were suspended in 25 mM Tris-HCl buffer (pH 8.5) containing 10 mM 2-mercaptoethanol, 1 mM EDTA, 0.1 mM phenylmethanesulfonyl fluoride, and 1 μg/mL phosphoramidon. Lysozyme (2 mg/mL) was added to the cell suspension, and then the mixture was stirred for 2 h at room temperature. The lysate was treated with deoxyribonuclease (0.1 mg/mL) at 4 °C for 1 h. Subsequent procedures were different for each component as follows.

The pHAEX01/JM109 lysate was centrifuged at 4000*g* for 30 min, and the pellet was washed twice with 25 mM Tris-HCl buffer (pH 7.5) containing 30 mM NaCl. After another centrifugation at 6000*g* for 10 min, the pellet was suspended in 25 mM Tris-HCl buffer (pH 7.5) containing 1 mM EDTA, and then the suspension was treated with urea to a final concentration of 8 M and stirred at room temperature for 20 min. The resulting solution was dialyzed against 25 mM Tris-HCl buffer (pH 8.5) containing 10 mM 2-mercaptoethanol and 1 mM EDTA (buffer A), with five changes of a decreasing concentration of urea (6 → 4 → 2 → 1 → 0 M) at 4 °C for a total of up to 12 h. The renatured component I (GerC1) was chromatographed on a DEAE-Toyopearl column (2.5 cm × 50 cm) equilibrated with buffer A with elution of a linear gradient of 0 to 1.0 M NaCl in buffer A. The component I fractions were collected, dialyzed against buffer A, and then applied to a Mono Q HR16/10 column (Pharmacia Biotech Inc.) equilibrated with buffer A. The column was eluted with a linear gradient of 0 to 0.5 M NaCl in buffer A, and the fractions containing component I were combined and dialyzed against 25 mM Tris-HCl buffer (pH 7.1) containing 10 mM 2-mercaptoethanol (buffer B). Then the solution was applied to a Mono P HR 5/5 column (Pharmacia Biotech Inc.) equilibrated with buffer B. Elution was carried out with a pH gradient of 7 to 4 formed by Polybuffer 74 acting as an eluent to obtain homogeneously purified component I fractions.

The pHAEX03/JM109 lysate was centrifuged at 100000*g* for 1 h at 4 °C, and the protein fraction that precipitated between 30 and 80% saturation of ammonium sulfate was dialyzed against buffer A and chromatographed on a DEAE-Toyopearl column equilibrated with buffer A. The column was eluted with a linear gradient of 0 to 1.0 M NaCl in buffer A. The component II fractions were pooled, dialyzed against buffer A containing ammonium sulfate at 30% saturation (buffer C), and then loaded onto a butyl-Toyopearl column (2.5 cm × 10 cm) equilibrated with buffer C. Elution was started with the same buffer followed by a decreasing linear gradient of ammonium sulfate from 30 to 0% saturation. The component II fractions, after dialysis against buffer A, were applied to a Mono Q HR 16/10 column with elution of a linear gradient of 0 to 0.5 M NaCl in buffer A. The fractions containing component II were pooled, dialyzed against buffer B, and subsequently purified by Mono P HR 5/5 chromatography with elution conditions similar to those for the

purification of component I to obtain purified component II fractions.

Preparation of Antisera against the Two Recombinant Components and Immunoblotting Analyses. The antisera against the recombinant component I or component II of HepPP synthase were prepared separately by Hokudo Tohya Immunity Laboratory employing Bal b/a mice. A booster injection of 0.16 mg of the protein was given four times every 2 weeks after the first injection. The mice were bled, and the sera were obtained. The titer of each antiserum toward the enzymatic activity of HepPP synthase was examined by incubating various volumes of the antiserum solution with the corresponding component at 30 °C for 2 h, followed by centrifugation at 12000*g* for 10 min. The supernatants were then assayed for HepPP synthase activity by adding the counterpart component as described above.

Proteins were transferred from a SDS−PAGE gel to a nitrocellulose membrane (Schleicher & Schuell). The membrane was then incubated with the antiserum, followed by a secondary incubation with AP-conjugated goat anti-mouse IgG and color development according to the manufacturer's instructions (ICN Pharmaceuticals Inc.). All blots were calibrated with prestained molecular mass markers (Bio-Rad).

Gel Filtration Analysis of the Interaction between the Two Components. A reaction mixture of 1 nmol of component I and 1 nmol of component II in 25 mM Tris-HCl buffer (pH 8.5) containing 10 mM 2-mercaptoethanol and 1 mM Mg^{2+} (buffer D), in a final volume of 200 μL, was incubated at 30 °C for 30 min in the presence or absence of indicated concentrations of IPP, inorganic pyrophosphate (PP_i), or FPP and then subjected to a Superdex 200 HR 10/30 column (Pharmacia Biotech Inc.) equilibrated with 25 mM Tris-HCl buffer (pH 8.5) containing 10 mM 2-mercaptoethanol and 150 mM NaCl. The columm was eluted with the same buffer system at a flow rate of 0.3 mL/min, and the eluate was collected in 300 μL fractions. Aliquots (50 μL) were assayed for the component I and II activities separately with supplements of the counterpart component.

Other aliquots (20 μL) of fractions were subjected to SDS−PAGE and transferred to nitrocellulose filters, and the proteins were identified by immunoblotting analysis as described above. The Superdex 200 column was calibrated with the following gel filtration molecular mass standards (Pharmacia Biotech Inc.): aldolase (158 kDa), albumin (67 kDa), ovalbumin (43 kDa), chymotrypsinogen A (25 kDa), and ribonuclease A (13.7 kDa).

Cross-Linking of the Two Components. Each mixture containing, in a final volume of 20 μL, 20 mM sodium phosphate buffer (pH 7.5), 150 mM NaCl, 10 mM 2-mercaptoethanol, 1 mM $MgCl_2$, and 2 μg of each component, I and II, was incubated at 30 °C for 15 min in the presence or absence of 5 μM IPP, PP_i, or FPP. To each of the resulting mixtures was added disuccinimidyl suberate (DSS, Pierce Chemical Co.) at a final concentration of 2.5 mM. After incubation for 30 min at room temperature, the cross-link reaction was stopped by addition of 0.1 M Tris-HCl buffer (pH 7.5) and the mixture was incubated at room temperature for an additional 15 min. The samples were separated on a 12% mini SDS−polyacrylamide gel, transferred to a nitrocellulose filter, and subjected to immunoblotting analysis (see above).

Assay of Complex Formation between the Two Components by Gel Filtration with Radiolabeled Substrates. A mixture (200 μL) containing 8 nmol of [1-^{14}C]IPP (1.95 TBq/mol), 10 nmol of nonlabeled FPP or 7.5 pmol of [1-^{3}H]FPP (2.22 TBq/mmol), and 5 nmol of nonlabeled IPP in buffer D was incubated at 30 °C for 30 min in the absence or presence of components I and II (each at 2 μM), and the resulting mixture was applied to a Superdex 200 HR 10/30 column equilibrated with buffer D. The column was eluted with the same buffer at a flow rate of 0.3 mL/min. Radioactivity in each fraction (0.3 mL) was measured.

Photoaffinity Labeling Experiments. Component I (5 μg), component II (4 μg), or a combination of components I (5 μg) and II (4 μg) was preincubated with 50 μM [1-^{3}H]-*m*-Bz-C_{10}-OPP for 10 min at room temperature in 25 mM Tris-HCl buffer (pH 8.5) containing 25 mM NH_4Cl, 10 mM 2-mercaptoethanol, and 1 mM $MgCl_2$ in a total volume of 100 μL. The resulting solutions were placed in wells of a polystyrene plate under a UV lamp (6 W, 360 nm) held at a distance of 2−3 cm, cooled to 4 °C, and irradiated for 20 min. After concentration with Microcon 10 concentrators (Amicon), the samples were mixed with 4× Laemmli sample buffer and subjected to SDS−PAGE on a 12% gel with prestained protein standards (Bio-Rad). The proteins were transferred from the SDS−PAGE gel to a nitrocellulose membrane, and the membrane was exposed on a Fuji imaging plate for 2 weeks. The distribution of radioactivity was analyzed with a Fuji BAS 1000 Mac bio-imaging analyzer.

RESULTS

Construction of a Plasmid Harboring the gerC Gene Region by Colony Hybridization. As there were several nucleotide replacements in *gerC1* and *gerC3* gene fragments obtained by PCR in our previous report (*6*), we intended to obtain an intact *gerC* gene region from a genomic DNA library of *B. subtilis* by an alternative colony hybridization. In consideration of the DNA sequences reported by Yazdi and Moir (*21*), we inserted approximately 6 kbp chromosomal DNA fragments obtained by digestion with *Fba*I into the *Bam*HI site of pUC 119, and the resulting plasmids were used for transformation of *E. coli* JM109 cells to construct a genomic library of *B. subtilis*. The library was screened for positive colonies that hybridized with the PCR-amplified probe containing the mutated *gerC1* or *gerC3* region. Positive colonies were then examined for expression of HepPP synthase activity, and a plasmid (pHEP02) which carries a 6.2 kbp DNA fragment was isolated. Then a 2.9 kbp fragment was obtained from pHEP02 by *Xba*I digestion, and the fragment was subcloned into pUC119 to give a plasmid, pHEP04 (Figure 1).

Construction of Expression Vector Systems for HepPP Synthase. To construct an expression plasmid for *gerC1* or *gerC3*, we subcloned the DNA fragments containing *gerC1* or *gerC3* into pUC119 separately and obtained two plasmids, pSH01 or pSE01, respectively. After introduction of several restriction sites immediately upstream and downstream of *gerC1* and *ger*C*3*, respectively, each of the gene fragments was subcloned into pTrc99A to construct a *gerC1* expression plasmid (pHAEX01) and a *gerC3* expression plasmid (pHAEX03) (Figure 1).

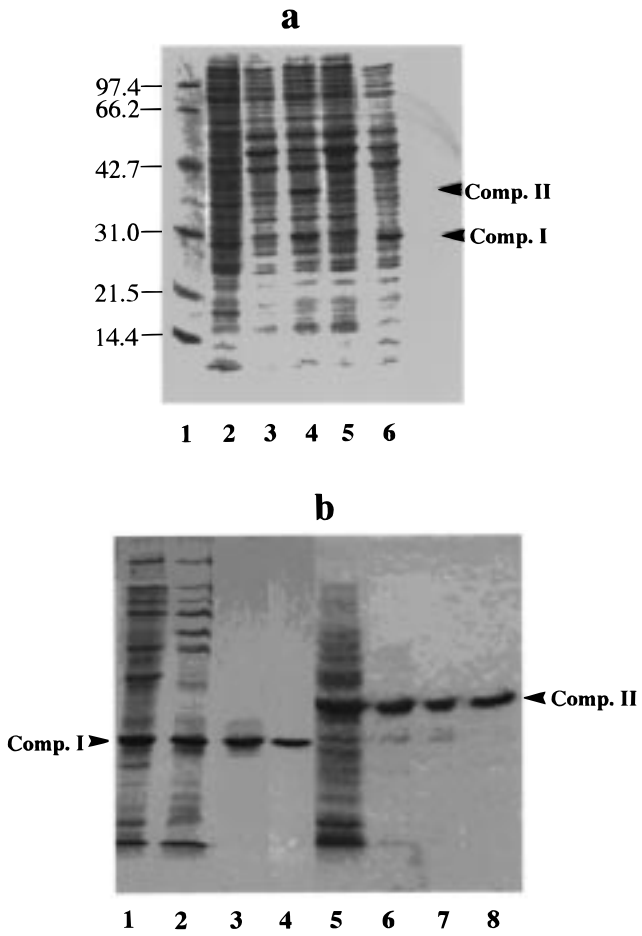

FIGURE 2: SDS−PAGE (14%) analyses of overproduction (a) and purification (b) of components I and II from *E. coli* cells harboring pHAEX01 or pHAEX03. (a) Total protein extracts from *E. coli* JM109 transformants: lane 1, molecular mass markers; lane 2, *E. coli* JM109/pTrc99A; lane 3, *E. coli* JM109/pHAEX03 (without IPTG); lane 4, *E. coli* JM109/pHAEX03 (with IPTG); lane 5, *E. coli* JM109/pHAEX01 (without IPTG); and lane 6, *E. coli* JM109/pHAEX01 (with IPTG). (b) Purification of component I or component II: lane 1, insoluble pellet of JM109/pHAEX01 (with IPTG); lane 2, after DEAE-Toyopearl chromatography of renatured pellet fraction; lane 3, after Mono Q chromatography of component I; lane 4, after Mono P chromatography of component I; lane 5, after DEAE-Toyopearl chromatography of JM109/pHAEX03 (with IPTG); lane 6, after butyl-Toyopearl chromatography of component II; lane 7, after Mono Q chromatography of component II; and lane 8, after Mono P chromatography of component II. Arrowheads indicate the locations of each component.

Overproduction and Purification of the Two Components of HepPP Synthase. As shown in Figure 2a, SDS−PAGE of the lysate of *E. coli* cells harboring pHAEX01 or pHAEX03 showed a thick band of component I at 29 kDa (lane 6) or of component II at 36 kDa (lane 4), respectively.

As the component I overproduced in *E. coli* cells formed inclusion bodies (Figure 2b, lane 1), we carried out renaturation of the component with 8 M urea, followed by stepwise dialyses. After renaturation, component I was purified by two steps of ion-exchange chromatography followed by isoelectric focusing, in which component I eluted at pH 5.1. Component II was purified by four chromatographic procedures, including isoelectric chromatography (p*I* value of 5.2). As shown in Figure 2b, each component was purified homogeneously (lanes 4 and 8).

Characterization of the Recombinant HepPP Synthase. The recombinant enzyme as a combined mixture of components I and II exhibited a broad pH optimum of 7.5−9.0 and required a divalent cation for the enzymatic activity with an optimal level of 1 mM Mg^{2+} or 2 mM Mn^{2+}, respectively. The substrate specificity of the recombinant enzyme was examined with respect to several allylic diphosphates as cosubstrates with IPP. (all-*E*)-FPP and (all-*E*)-GGPP are both good substrates, whereas short-chain allylic diphosphates, dimethylallyl diphosphate (DMAPP) and geranyl diphosphate (GPP), are almost inactive as substrates. Initial velocities were measured for varied concentrations of one substrate in the presence of a fixed concentration of the other (50 μM IPP or 100 μM FPP) in the standard assay mixture. Michaelis constants, calculated from the Lineweaver−Burk plots, were 7.1 ± 0.9, 8.5 ± 1.2, and 16.7 ± 1.8 μM for FPP, GGPP, and IPP, respectively. One nanomole of the recombinant enzyme (a combination of 1 nmol of component I and 1 nmol of component II) converts 1.10 μmol of IPP to products per minute for the reaction with FPP.

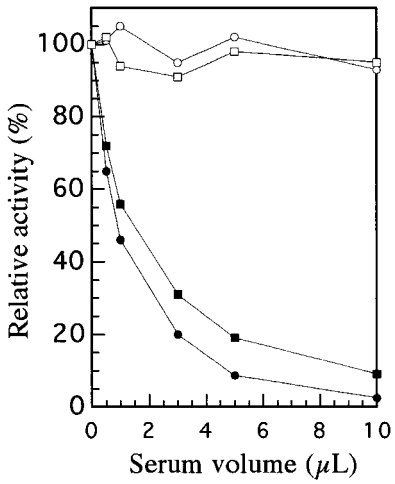

FIGURE 3: Inhibition of the enzymatic activity of HepPP synthase by the antiserum against one of the two components. The indicated amount of preimmune serum or antiserum was incubated with the respective component, component I (1 μg; ○, with preimmune serum; ●, with anti-GerC1) or component II (1 μg; □, with preimmune serum; ■, with anti-GerC3), and the residual enzymatic activity of each component was assayed by adding the corresponding counterpart component (1 μg) as described in Experimental Procedures.

Inhibition of the Enzyme Activity of HepPP Synthase by Antisera. The purified components were used for preparation of antisera, and titers of the antisera were evaluted in terms of inhibitory effects on the HepPP synthase activity. The enzymatic activity was inhibited by each antiserum against component I or component II as shown in Figure 3, indicating that either component I or component II is confirmed immunochemically to be essential for enzymatic activity.

Interaction between the Two Essential Components. It is interesting to investigate the interaction between the two components during enzymatic catalysis. Figure 4 shows gel filtration profiles of mixtures of components I and II preincubated in the absence or presence of substrates, FPP, IPP, or the other product, PP_i. In the absence of these compounds, component I and component II eluted at positions corresponding to molecular masses of 29 and 36 kDa, respectively (panel a), which were identical with those observed when they were chromatographed individually (data not shown). The enzymatic activity peak around fraction

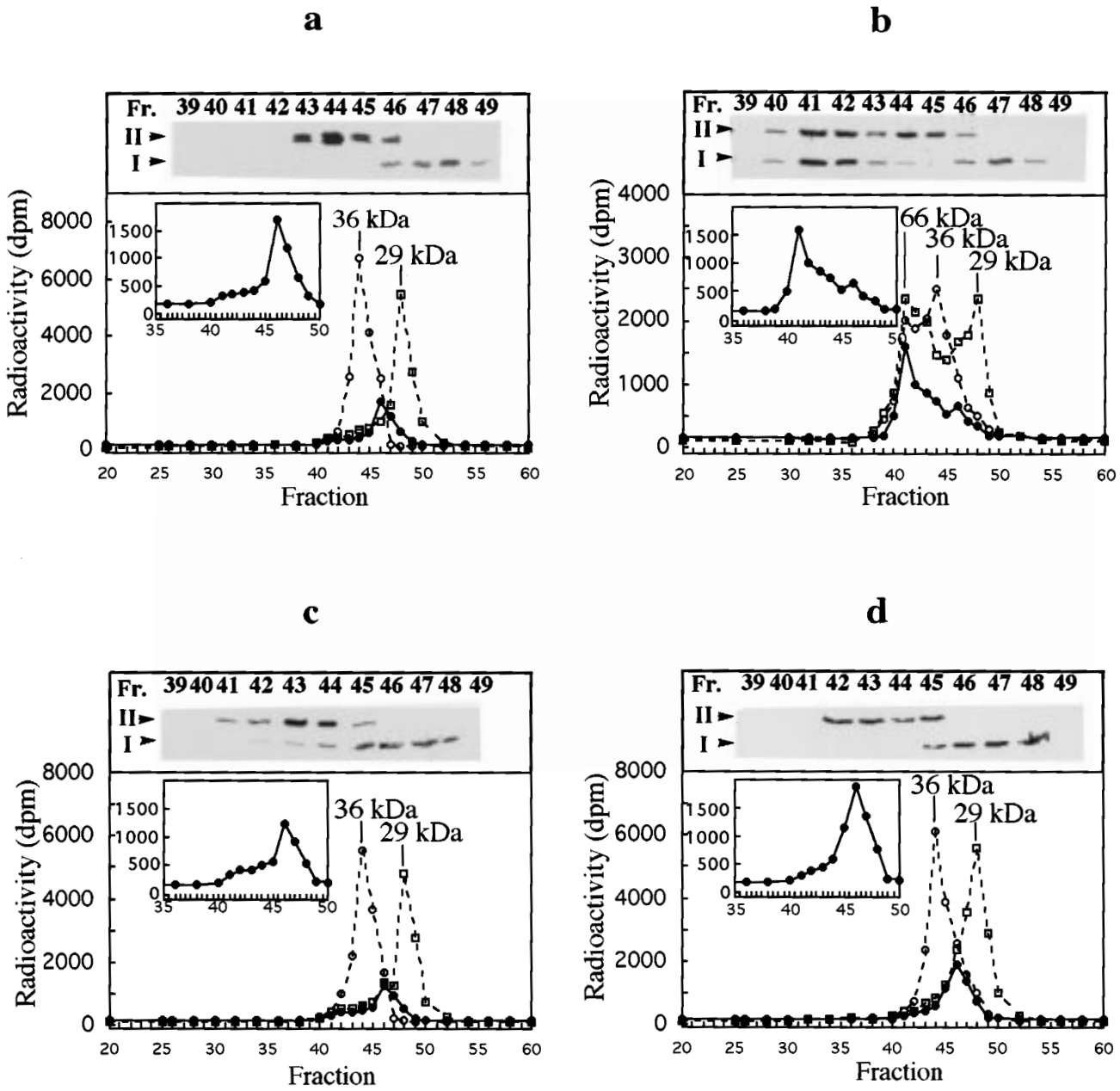

FIGURE 4: Interaction between the two essential components. Each reaction mixture (200 μL) of component I (1 nmol) and component II (1 nmol) in buffer D was incubated with no substrates (a), with 20 μM FPP (b), with 20 μM IPP (c), or with 20 μM PP_i (d) at 30 °C for 30 min and then applied to a Superdex 200 HR column as described in Experimental Procedures. Fifty microliter aliquots of each fraction (300 μL) were assayed for the enzymatic activity without supplement of components (●), with a supplement of component I (○), or with component II (□). Insets show magnified illustrations of fractions 35−50. Aliqouts (20 μL) of fractions 39−49 were subjected to SDS−PAGE on 12% mini gels, and immunoblot analyses were carried out with anti-component I or anti-component II serum as described in Experimental Procedures. Arrowheads indicate the positions of components I and II on nitrocellulose membranes. Fr. represents fraction.

46 that is detectable without the supplement of the counterpart component could be attributed to overlapping of the two components without interaction. Although a wide range of broad activity peaks around fractions 41−45 was also observed, no protein that could be attributed to the association between the two components was detected by immunoblotting (panel a, upper box). On the other hand, in the presence of FPP and Mg^{2+}, a significant activity peak was eluted at a position corresponding to a molecular mass of 66 kDa (panel b). This value is in accord with the sum of the molecular mass values of components I and II. Furthermore, approximately 50% of the total amount of both components added in the preincubation mixture was detected in this peak by immunoblotting analysis (panel b, upper box). This peak seemingly can be attributed to a catalytically active state of the two components. In contrast, neither a similar peak nor migration of a significant amount of each component in this elution region was found in panels c and d, where the mixtures were preincubated in the presence of Mg^{2+} and IPP (panel c) or PP_i (panel d). These results suggest that the allylic substrate, FPP, is required for the association between the two essential components.

Formation of a Complex of the Components and Substrates during Enzymatic Catalysis. Figure 5 shows the result of the experiment in which mixtures of components I and II were incubated with a cross-linker (DSS) in the absence or presence of Mg^{2+} and IPP, PP_i, or FPP. The cross-linking reaction mixtures were then analyzed by SDS−PAGE,

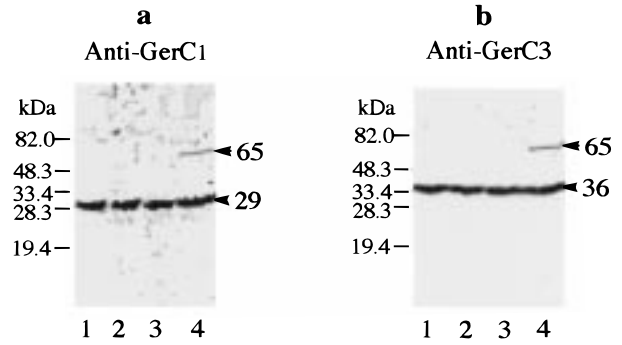

FIGURE 5: Cross-linking of the two components. Each cross-linking reaction was carried out as described in Experimental Procedures. After cross-linking, the samples were subjected to SDS−PAGE, transferred to nitrocellulose paper, and subjected to immunoblot analysis with each antiserum directed against component I (a) or component II (b): lane 1, mixture of components I and II without substrates or PP_i; lane 2, in the presence of Mg^{2+} and IPP; lane 3, in the presence of Mg^{2+} and PP_i; and lane 4, in the presence of Mg^{2+} and FPP. The positions of molecular mass standards are shown by bars at the left. Arrowheads indicate the positions of components detected by immunoblotting.

followed by blotting with each antiserum against component I (panel a) or component II (panel b). Only the incubation in the presence of Mg^{2+} and FPP showed a weak but significant band that could be detected with either of the antisera at 65 kDa (lanes 4 in panels a and b), suggesting that the two components were cross-linked with each other.

The association between the two components was further studied by radiochromatographic analysis on Superdex 200. Panel a in Figure 6 shows the chromatogram of the two components preincubated in the presence of FPP, [1-^{14}C]-IPP, and Mg^{2+}. The radioactivity fractions in the small peak at the V_0 region were collected and analyzed after acid phosphatase treatment. As shown in panel c of Figure 6, only C_{35}-alcohol, derived from the enzymatic reaction product, HepPP, was detected, indicating that HepPP was eluted as micellar aggregates at the V_0 region in gel filtration. The radioactivity peaks at fractions 50−66 could be attributed to recovered [1-^{14}C]IPP. No radioactivity peak was detected in the other regions. When the mixture was preincubated in the presence of [1-^{3}H]FPP, nonlabeled IPP, and Mg^{2+}, a small but significant peak of radioactivity was observed at the elution position of fractions 40−42, where the 66 kDa complex was eluted (panel b), in addition to the peaks at the V_0 region and the V_t region due to HepPP and recovered FPP, respectively. These results confirm that component I, component II, and FPP-Mg^{2+} form a ternary complex during catalysis and that neither IPP nor the product, HepPP, is included in this complex, which probably represents a catalytically active state of the HepPP synthase.

Photoaffinity Labeling of the HepPP Synthase with [1-^{3}H]-m-Bz-C_{10}-OPP. An effort to examine the active sites that bind the allylic substrate FPP between the two components was made by employing a photoaffinity analogue of this substrate. Marecak et al. (*15*) have reported that a photoactivatable analogue of FPP, *m*-Bz-C_{10}-OPP, was effective as a photoaffinity labeling reagent for several prenyltransferases. This analogue has been shown to behave not only as an active site-directed competitive ligand when incubated with *B. subtilis* HepPP synthase but also as a substrate, showing a relative activity that is 23.2% of that of FPP (*22*).

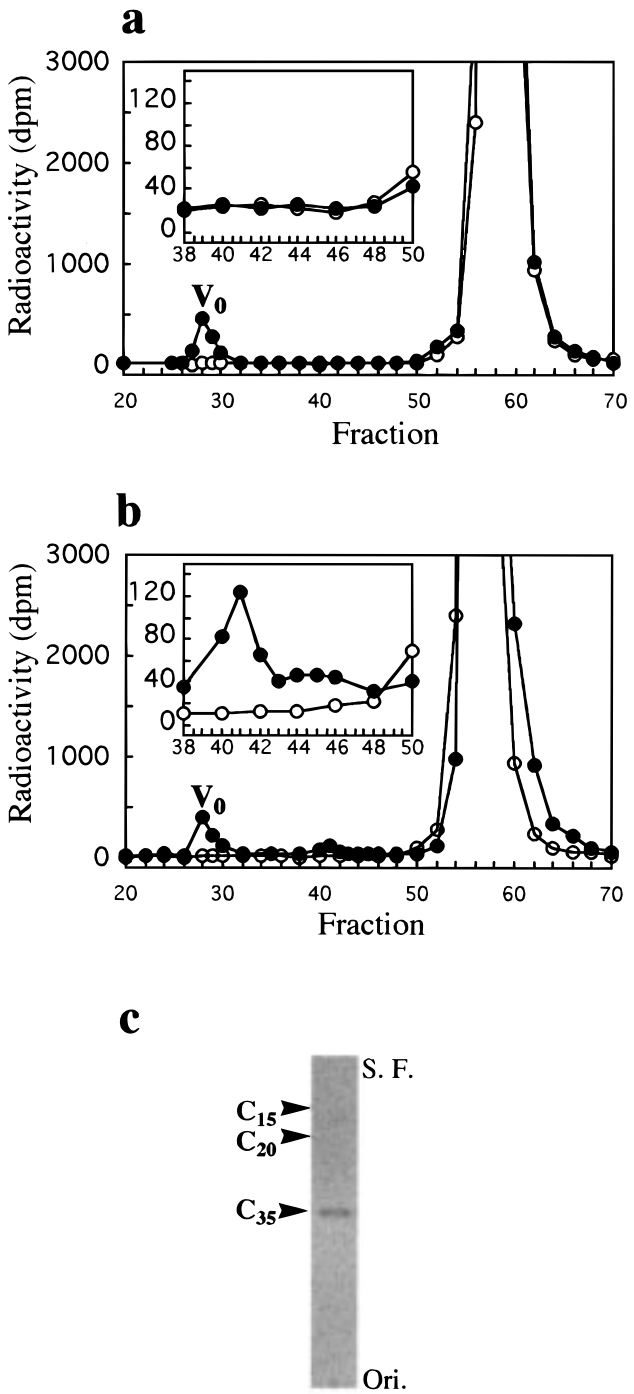

FIGURE 6: Formation of the ternary complex during catalytic reaction. (a) A reaction mixture containing [1-^{14}C]IPP and nonlabeled FPP in buffer D was incubated at 30 °C for 30 min in the absence (○) or presence of 2 μM component I and II (●) and then applied to a Superdex 200 HR column as described in Experimental Procedures. Each fraction (300 μL) was evaluated for ^{14}C radioactivity. (b) A reaction mixture containing [1-^{3}H]FPP and nonlabeled IPP in buffer D was incubated at 30 °C for 30 min in the absence (○) or presence of 2 μM component I and II (●) and then chromatographed as described in Experimental Procedures. Each fraction (300 μL) was evaluated for ^{3}H radioactivity. Insets show magnified illustrations of the elution profile of radioactivity from fractions 38−50. (c) TLC autoradiogram of the alcohol obtained dy enzymatic hydrolysis of the radioactivity fractions in the V_0 region of panel a. Radioactivity fractions 27−30 in the V_0 region were combined, and the product analysis was carried out as described in Experimental Procedures. Arrowheads indicate the positions of authentic alcohols: C_{15}, (all-*E*)-farnesol; C_{20}, (all-*E*)-geranylgeraniol; C_{35}, (all-*E*)-heptaprenol; Ori, origin; and S. F., solvent front.

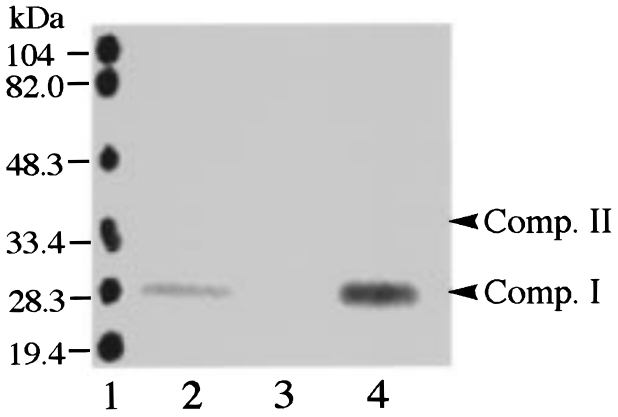

FIGURE 7: Photoaffinity labeling of the HepPP synthase with [1-^{3}H]-*m*-Bz-C_{10}-OPP. Each component or a mixture of the both was irradiated with 50 μM [1-^{3}H]-*m*-Bz-C_{10}-OPP (129.5 MBq/mmol) in 25 mM Tris-HCl buffer (pH 8.5) containing 25 mM NH_4-Cl, 10 mM 2-mercaptoethanol, and 1 mM $MgCl_2$ for 20 min at 4 °C. The proteins were subjected to SDS−PAGE on a 12% gel and then transferred to a nitrocellulose membrane. The distribution of radioactivity was determined by autoradiography: lane 1, molecular mass marker as marked with radioactivity; lane 2, component I (5 μg); lane 3, component II (4 μg); and lane 4, a mixture of component I (5 μg) and component II (4 μg). Arrowheads indicate the positions of component I and component II on the membrane.

Figure 7 shows the result of photoaffinity labeling of the HepPP synthase with [1-^{3}H]-*m*-Bz-C_{10}-OPP. The photoaffinity probe showed a weak but specific affinity for the component I protein, but the level of photolabeling of component I significantly increased when component II was present (Figure 7, lane 4). In the absence of irradiation, no radioactive bands were observed (data not shown). These results suggest that component I is involved in allylic substrate recognition.

DISCUSSION

The HepPP synthase from *B. subtilis* was purified partially as a mixture of the two components to 15-fold from crude cell homogenate and characterized (*4*). Subsequently, Fujii et al. (*5*) first reported that the enzyme comprises two essential components, components I and II, and that the enzymatic activity required both components.

In this work, we purified each of the two recombinant components of HepPP synthase of *B. subtilis* to homogeneity and used them to reconstitute the enzymatic activity. As a result, most of the characteristics of the recombinant enzyme indicate that the enzyme is almost identical with the native synthase from *B. subtilis*.

The data presented herein indicate that the two subunits of HepPP synthase in the presence of FPP and Mg^{2+} form a stable ternary complex, I−II−Mg^{2+}-FPP, which exhibits an apparent molecular mass of 66 kDa. This was detected and confirmed by gel filtration (Figure 4b) and cross-linking experiments (Figure 5). On the other hand, the two components were not associated with each other in the absence of FPP, and no significant elution of such a complex was observed at the 66 kDa position. Several lines of evidence show that neither the homoallylic substrate, IPP, nor the products, HepPP and PP_i, are included in the active ternary complex (Figure 4, panels c and d; Figure 6, panel c). Hence, it is likely that FPP is essential for the formation of this complex, which seems to represent an intermediary state of the enzymatic catalysis. The gel filtration in panel b of Figure 4 shows a significant activity peak with a molecular mass of 66 kDa. This value is approximately consistent with the sum of the molecular mass values of the two components. The fractions at the elution position of 66 kDa were demonstrated to contain both components I and II by immunoblotting analysis (Figure 4b, upper box). Furthermore, the two components became covalently linked by following incubation with a cross-linker (DSS) in the presence of Mg^{2+} and FPP, and the cross-linked form showed a band at 65 kDa on SDS−PAGE (Figure 5). These results reveal that the two components tend to associate with each other in a 1/1 ratio in the presence of FPP and Mg^{2+}.

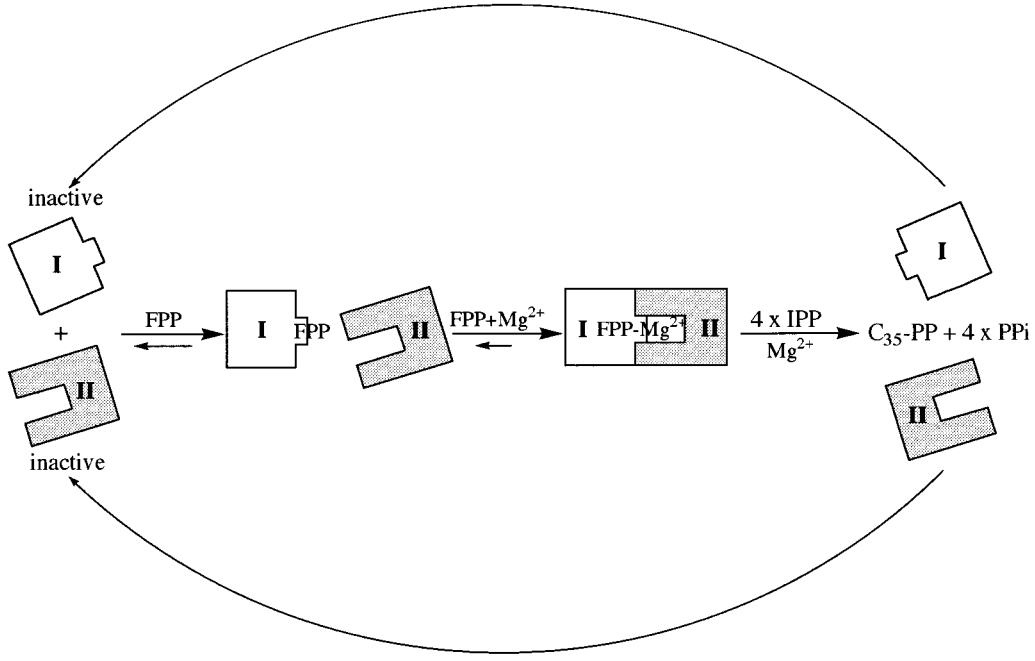

FIGURE 8: Hypothetical mechanism of the formation and dissociation of the catalytically active complex of HepPP synthase of *B. subtilis*.

Solanesyl diphosphate (SPP) synthase of *M. luteus*, is a nondissociable homodimeric enzyme showing a weak but significant catalytic activity by itself. However, it is markedly stimulated by a protein factor that removes the polyprenyl products from the active site of the enzyme (*2*). The protein factor stimulates (*E*)-octaprenyl- and (*E*)-decaprenyl diphosphate synthases from *E. coli* (*23*) and *Paracoccus denitrificans* (*24*), respectively, as well as the SPP synthase, but it has no effect on undecaprenyl [(*E*)-farnesyl-(*Z*)-octaprenyl] diphosphate synthase, which is also a homodimeric enzyme (3). Therefore, this protein factor acts specifically on long-chain (*E*)-prenyl products with 40, 45, and 50 carbons. In this context, it has been proposed that this protein factor should be designated as a polyprenyl carrier protein (*1*). Component I of the HepPP synthase does not seem to have a function similar to that of the carrier protein for the long-chain (*E*)-prenyl diphosphate for the following reasons. The enzymatic product, HepPP, was found to elute as micellar aggregates at the V_0 region in gel filtration (Figure 6, panels a and c), while long-chain prenyl products such as SPP were known to elute as a bound form with the carrier protein (*2*). Although several lines of evidence indicated that component II of HepPP synthase carries the major sites for substrate binding and catalytic activity, this component has no prenyltransferase activity alone unless it is combined with component I (*5*, *6*). In addition, component II shows no catalytic activity even in the presence of various kinds of detergents such as Triton X-100, Tween 80, CHAPS, and octyl glycoside or the polyprenyl carrier proteins, BSA or bacitracin, all of which stimulate SPP synthase (data not shown). The components of *M. luteus* HexPP synthase and those of *B. subtilis* HepPP synthase are so specific for their own partners that none of the components can substitute for any of the heterologous counterparts (*5*).

The three-dimensional structure of avian liver FPP synthase shows that the synthase is a homodimer of a subunit containing a large cavity that involves the catalytic site (*25*). Two aspartate-rich motifs (DDXXD), which are essential and highly conserved among (*E*)-prenyl diphosphate synthases (*26*−*28*), are located in paired helices that run nearly antiparallel on opposite walls of this cavity (*25*). Though the HepPP synthase of *B. subtilis* is a heterodimer composed of dissociable components (components I and II), component II possesses motifs similar to those of avian liver FPP synthase, including two aspartate-rich motifs typical of (*E*)-prenyl diphosphate synthases. It is therefore reasonable to assume that component II has a tertiary structure similar to the subunit of the avian FPP synthase. Recently, the crystal structure of avian FPP synthase with allylic substrates bound has been analyzed (*29*), and it has been shown that allylic diphosphates bind through magnesium ions to the aspartates of the conserved aspartate-rich sequences (DDXXD) with the hydrocarbon tails of the ligands growing down the hydrophobic pocket.

These data show that FPP is essential for the formation of the active complex of the HepPP synthase and that IPP or PP_i is not essential. This fact may raise a possibility that the hydrocarbon tail of FPP binds to hydrophobic binding site(s) that is formed by the association between the two subunits. Thus, it is assumed that the FPP-assisted association between the two subunits requires a scenario that involves transient dimerization that is then stabilized by binding of FPP-Mg^{2+}. However, we have no direct evidence to support the assumption that the two components would associate to form a transient dimer by some specific interactions between them. Furthermore, photoaffinity labeling experiments contradict the possibility and assumption in which [1-^{3}H]-*m*-Bz-C_{10}-OPP, a photoreactive analogue of FPP that contains a benzophenone group positioned at the tail of the prenyl chain, became photocovalently attached to the component I protein even when this subunit was present alone (Figure 7, lane 2). This suggests that component I participates in binding of the allylic substrate, most likely through the hydrophobic prenyl tail of the allylic substrate. It should be noted that the level of photoaffinity-labeled component I in the presence of component II significantly increased in contrast to that in the absence of the component (Figure 7, lanes 2 and 4). Therefore, it is reasonable to assume that the two components with the allylic substrate analogue form a stable ternary complex, and the component I−photoprobe association is enhanced by the presence of component II. Taken together, it seems that a more likely scenario for the formation of the complex involves binding of FPP-Mg^{2+} with component I of the two subunits first and the subsequent association of component II that then stabilizes the complex, possibly through Mg^{2+} that coordinately binds the diphosphate moiety of allylic substrate. This gives a mechanism for the association and dissociation of the catalytically active complex as HepPP synthase (Figure 8). Component I first binds the prenyl chain of the allylic substrate FPP and then associates with component II in the presence of Mg^{2+} to form a stable ternary complex, I−II−Mg^{2+}-FPP. This complex catalyzes the consecutive condensation of FPP and four molecules of IPP to produce HepPP. Then the complex dissociates into the two components with concomitant release of the product, HepPP, which finally forms a micelle in an aqueous phase because of its amphipathic nature. Thus, the two subunits will repeat this association−dissociation process. This dynamic process might account for the ability of this unique enzyme to make an efficient turnover of synthesis of amphipathic products from soluble substrates without the aid of detergent-like molecules.

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