Mutational Analysis of Allylic Substrate Binding Site of *Micrococcus luteus* B-P 26 Undecaprenyl Diphosphate Synthase†

Keitaro Fujikura,‡ Yuan-Wei Zhang,§ Masahiro Fujihashi,|| Kunio Miki,||,⊥ and Tanetoshi Koyama*,‡

Institute of Multidisciplinary Research for Advanced Materials, Tohoku University, Katahira 2-1-1, Aoba-ku, Sendai 980-8577, Japan, the Department of Chemistry, Graduate School of Science, Kyoto University, Sakyo-ku, Kyoto 606-8502, Japan, and the RIKEN Harima Institute/SPring-8, Koto 1-1-1, Mikazukicho, Sayo-gun, Hyogo 679-5148, Japan

Received December 4, 2002; Revised Manuscript Received February 13, 2003

ABSTRACT: Undecaprenyl diphosphate (UPP) synthase catalyzes the sequential cis-condensation of isopentenyl diphosphate (IPP) onto (*E*,*E*)-farnesyl diphosphate (FPP). In our previous reports on the *Micrococcus luteus* B-P 26 UPP synthase, we have shown that the conserved residues in the disordered region from Ser-74 to Val-85 is crucial for the binding of FPP and the catalytic function [Fujikura, K., et al. (2000) *J. Biochem. (Tokyo) 128*, 917−922] and the existence of a structural P-loop motif for the FPP binding site [Fujihashi, M., et al. (2001) *Proc. Natl. Acad. Sci. U.S.A., 98*, 4337−4342]. To elucidate the allylic substrate binding site in more detail, we prepared eight mutant enzymes and examined their kinetic behavior. The mutant with respect to the two complementarily conserved Arg residues among the structural P-loop motif, G32R-R42G, retained the activity and showed product distribution pattern exactly similar to that of the wild-type, indicating that the complementarily conserved Arg is important for maintaining the catalytic function. Substitutions of Asp-29, Arg-33, or Arg-80 with Ala resulted in a large loss of enzyme activity, suggesting that these residues are essential for catalytic function. However, the K_m values of these mutant enzymes for *Z*-GGPP, which is the first intermediate during the enzymatic cis-condensations of IPP onto FPP, were only moderately different or little changed from those of the wild type. These results suggest that the binding site for the intermediate *Z*-GGPP having a cis double bond is different to that for the intrinsic allylic substrate, FPP, whose diphosphate moiety is recognized by the structural P-loop.

Prenyltransferases (so-called prenyl diphosphate synthases) catalyze fundamental isoprenoid chain elongation to produce prenyl diphosphates with various chain lengths and stereochemistries, which are converted to such diverse isoprenoid compounds as steroids, carotenoids, glycosyl carrier lipids, prenyl quinones, and prenylated proteins in the biosynthetic pathway of isoprenoids. These enzymes can be classified into two major subgroups (cis- and trans-types) according to the geometry of their products that are determined by the distinct specificity of each enzyme.

The molecular mechanism of prenyltransferases catalyzing the formation of *trans*-prenyl diphosphates have been intensively studied (*1*, *2*). The amino acid sequence alignment of the trans-type prenyltransferases shows the presence of several conserved regions including two characteristic aspartate-rich motifs (DDXXD), which have been shown to be essential for catalysis and substrate binding by site-directed mutational analyses (*3*−*6*). Tarshis et al. (*7*) have determined the crystal structure of avian farnesyl diphosphate (FPP) synthase, in which most of the conserved regions are found in a large central cavity. Furthermore, analysis of the three-dimensional structure of the FPP synthase bound with substrates has revealed that the enzyme uses the Asp residues in the DDXXD motifs to bind the diphosphate groups of the substrates through Mg^{2+} bridges (*8*).

On the other hand, cis-type prenyltransferases catalyze *cis*-prenyl chain elongation to form *cis*-prenyl diphosphates with *E*,*Z*-mixed stereochemistry. Undecaprenyl diphosphate (UPP)[1] synthase catalyzes sequential cis-condensation of eight molecules of isopentenyl diphosphate (IPP) onto FPP as an allylic primer to produce UPP (C_{55}), which is involved in the biosynthesis of the bacterial cell wall. Dehydrodolichyl diphosphate synthase, a cis-type prenyltransferase in eukaryotic cells, is responsible for the formation of dehydrodolichyl diphosphate, a precursor of the sugar carrier lipid dolichol, in the biosynthesis of N-linked glycoprotein or glycosylphos-

† This work was supported in part by Grants-in-Aid for Scientific Research (12480169 to T.K. and 13680667 to Y.-W.Z.) from the Ministry of Education, Science, and Culture of Japan, by the "Research for the Future" Program (JSPS-RFTF 97I00302 to T.K.) from the Japan Society for the Promotion of Science, by the Asahi Glass Foundation, by the Heiwa Nakajima Foundation, and by the Sumitomo Foundation.

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

‡ Tohoku University.

§ Current address: Department of Pharmacology, Yale University, 333 Cedar St., New Haven, CT 06520.

|| Kyoto University.

⊥ RIKEN Harima Institute/SPring-8.

[1] Abbreviations: BSA, bovine serum albumin; FP, (*E*,*E*)-farnesyl monophosphate; FPP, (*E*,*E*)-farnesyl diphosphate; *Z*-GGPP, (*Z*,*E*,*E*)-geranylgeranyl diphosphate; (all-*E*)-GGPP, (*E*,*E*,*E*)-geranylgeranyl diphosphate; IPP, isopentenyl diphosphate; IPTG, isopropyl β-D-thiogalactopyranoside; LB, Luria−Bertani; PCR, polymerase chain reaction; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; TLC, thin-layer chromatography; UPP, undecaprenyl diphosphate; PPi, inorganic pyrophosphate.

10.1021/bi027236v CCC: $25.00
Published on Web 03/19/2003

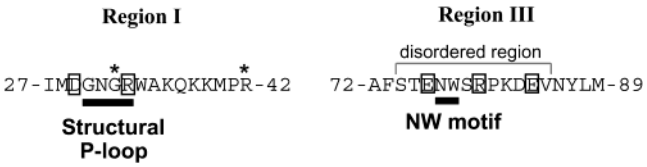

FIGURE 1: Brief description of the primary structures of the conserved Regions I and III of *M. luteus* B-P 26 UPP synthase. Conserved amino acid residues that were exchanged with other amino acids by site-directed mutagenesis in this study are boxed, the complementarily conserved amino acid residues are marked by asterisks, and the disordered region (*18*), structural P-loop motif (*16*), and NW motif (*18*) are also indicated.

phatidylinositol-anchored protein. Several gene sequences for bacterial UPP synthase and eukaryotic dehydrodolichyl diphosphate synthase have recently been available (*9*−*13*). The deduced amino acid sequences of these *cis*-prenyl chain-elongating enzymes show the presence of five highly conserved regions, which however, lack the DDXXD motif and are totally different from those of trans-type prenyltransferases. This fact may raise the possibility that cis-type prenyltransferases take quite a different mode for substrate binding and catalysis from that of trans-type prenyl chain elongating enzymes. The stereochemical course of cis- and trans-type prenyltransferase reactions are similar to each other in that the C−C bond is formed on the same side of the double bond of IPP as the C−H bond is cleaved (*14*, *15*). Hence, it is of particular interest to learn the similarity and difference in the molecular mechanism between cis- and trans-type prenyltransferases.

We have recently determined the crystal structure of *Micrococcus luteus* B-P 26 UPP synthase as the first three-dimensional structure among *cis*-prenyl chain elongating enzymes (*16*). The enzyme shows a novel protein fold, which is completely different from those of other isoprenoid biosynthesis-related enzymes. In the molecular surface, there is a large cleft where most of the conserved amino acid residues of cis-type prenyltransferases are located. At the entrance of this cleft is found a common motif for phosphate recognition called a structural P-loop motif (*17*). We proposed the motif, composed of Gly-30, Asn-31, Gly-32, and Arg-33, to be involved in the binding of the diphosphate moiety of allylic substrate FPP (*16*). In addition, by our random mutagenesis studies, we have also reported that the conserved motif Asn−Trp in the disordered region from Ser-74 to Val-85 in the cis-type prenyltransferase is involved in the binding of FPP as well as the catalytic function (*18*) (Figure 1). Meanwhile, Ko et al. determined the structure of *Escherichia coli* UPP synthase structure, which is essentially similar to that of *M. luteus* B-P 26 UPP synthase (*19*). To better understand the functional significance of above-mentioned motifs that is proposed to be the binding site for allylic substrates, we selected seven conserved amino acid residues related to these motifs of *M. luteus* B-P 26 UPP synthase for substitutions by site-directed mutagenesis and examined their effects on catalytic function of the enzyme. This paper describes further elucidation of allylic substrate binding site of the *cis*-prenyl chain elongating enzyme.

EXPERIMENTAL PROCEDURES

Materials and General Procedures. [4-^{14}C]IPP (2.12 TBq/mol) and [1-^{3}H]FPP (18.5 GBq/mol) were purchased from Amersham. Nonlabeled IPP, FPP, and (*Z*,*E*,*E*)-geranylgeranyl diphosphate (*Z*-GGPP) were synthesized according to the procedure of Davisson et al. (*20*). Farnesyl monophosphate (FP) was the same preparation as used in the previous work (*21*). Restriction enzymes and other DNA-modifying enzymes were from Takara Shuzo Co., Ltd. and Toyobo Co., Ltd. Potato acid phosphatase was a product of Sigma. *E. coli* B strain BL21 (DE3) was used as the host for expression of the target gene. Restriction enzyme digestions, transformations, and other standard molecular biology techniques were carried out as described by Sambrook et al. (*22*). Precoated reversed phase thin-layer chromatography (TLC) plates were purchased from Merck. Bacteria were cultured in Luria−Bertani (LB) or M9YG medium (*6*). Plasmid DNA for sequencing reactions was isolated from transformed *E. coli* JM109 cells using Quantum Prep Plasmid Miniprep kit (Bio-Rad). The sequencing reaction was performed by using Thermo Sequenase cycle sequencing kit (Amersham) with fluorescent labeled primers. The nucleotide sequences were determined by a dideoxy chain termination method (*23*) with a DNA sequencer (Li-COR, model 4200). All other chemicals were of analytical grade. The protein sequence database Swiss-prot was searched to find amino acid sequences similar to that of *M. luteus* B-P 26 UPP synthase.

Site-Directed Mutagenesis. The plasmid pMluUEX, which carries the wild-type gene of *M. luteus* B-P 26 UPP synthase, was the same preparation as described previously (*24*). Site-directed mutagenesis was performed according to the protocol of the GeneEditor in vitro site-directed mutagenesis system (Promega). The single-stranded wild-type UPP synthase gene, used as a template in the mutagenesis reaction, was prepared by R408 helper phage infection of *E. coli* JM109 cells (TaKaRa) harboring pMluUEX. The mutagenic oligonucleotides (20−30 bases long) were designed to produce the desired point mutations as follows: D29A, 5′-ggccatttccggccataatgattg-3′; R33A, 5′-tgttttgcccatgcgccatttccg-3′; G32R, 5′-gcccatcggcgatttccgtcc-3′; R42G, 5′-gtccttttatgccggcatttttttctg-3′, E76Q, 5′-cgagaccaattttgagttgaaaatgcg-3′; R80A, 5′-ttaacctcat*ccttagg*agcagaccaattt-3′; E84Q, 5′-gtaattaacctgat*ccttagg*acgaga-3′ (mismatched bases are underlined, newly created *Eco*81I restriction sites are italicized). For double mutation G32R-R42G, the mutagenic reaction was carried out by using both mutagenic primers for G32R and R42G with the same concentration. Introduction of the mutation was confirmed by sequencing the whole nucleotide sequence.

Preparation of UPP Synthase Mutants. Each of the mutated enzymes overproduced in *E. coli* BL21 (DE3) cells (Novagen) was purified essentially according to the purification procedure as described in our previous work (*18*) including three chromatographic steps. The fractions of the mutated enzyme were analyzed for purity by sodium dodecyl sulfate−polyacrylamide gel electrophoresis (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 (*25*) with bovine serum albumin (BSA) as a standard.

UPP Synthase Assay and Product Analysis. The enzyme activity was measured by determination of the amount of [4-^{14}C]IPP incorporated into butanol-extractable polyprenyl diphosphates. The standard incubation mixture contained, in

a final volume of 0.2 mL, 100 mM Tris-HCl buffer, pH 7.5, 0.5 mM $MgCl_2$, 10 μM FPP or *Z*-GGPP, 10 μM [4-^{14}C]IPP (37 MBq/mol), 0.05% (w/v) Triton X-100, and a suitable amount of enzyme solution. After incubation at 37 °C for 15 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.

In sulfate ion-inhibition experiments, the 0.2 mL incubations contained varying concentrations of substrates and ammonium sulfate in addition to the contents for the standard assay. For steady-state kinetic studies, the concentration of allylic substrate FPP/*Z*-GGPP or homoallylic substrate [4-^{14}C]IPP was varied, while the other substrate [4-^{14}C]IPP or FPP/*Z*-GGPP was kept constant at 200 μM, respectively. Calculation of kinetic parameters was performed using EnzymeKinetics software version 1.5 (Trinity Software).

To analyze the radioactive products in the reaction mixture, the prenyl diphosphates were hydrolyzed to the corresponding alcohols with potato acid phosphatase according to our method reported previously (*26*). The alcohols were extracted with pentane and spotted on reversed phase TLC plates (LKC-18, Whatman), which were then developed 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 by autoradiography. The TLC plates were exposed on a Fuji imaging plate at room temperature for 1 day and then 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.

Measurement of the Dissociation Constants for Substrates. The binding of the substrate FPP or IPP to the UPP synthase was measured essentially according to the method of Dolence et al. (*27*). To the mixture of [1-^{3}H]FPP (18.5 GBq/mol, 5−160 nM) in 100 mM Tris-HCl buffer, pH 7.5, containing 0.5 mM Mg^{2+} and 0.05% Triton X-100 (100 μL final volume) was added the wild-type UPP synthase (2.5 μM). The mixtures were incubated at room temperature for 15 min. An aliquot (10 μL) was removed from the tube and mixed with 3 mL of a liquid scintillation cocktail Clear-Sol I (Nacalai Tesque) to determine the total radioactivity of the sample. The remainder of the mixture was transferred to a preequilibrated membrane filter, Microcon YM-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 the enzyme was 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 Scatchard plot and a standard binding equation (*28*) to obtain a K_d value of the substrate for the UPP synthase.

The binding of IPP to a FP-containing UPP synthase solution was measured essentially similar to the case with FPP. The wild-type UPP synthase (2.5 μM) was added to [4-^{14}C]IPP (1.95 TBq/mol, 0.2−2 μM) in 100 mM Tris-HCl buffer, pH 7.5, containing 0.5 mM Mg^{2+} and 0.05% Triton X-100 in the absence or presence of 100 μM FP (100 μL final volume). Subsequent procedures were the same in the case of FPP (see above).

RESULTS

Inhibitory Effect of Sulfate, Pyrophosphate Ions, or FP. In the crystal structure of *M. luteus* B-P 26 UPP synthase, a sulfate ion was found to be bound to the structural P-loop motif consisting of the residues from Gly-30 to Arg-33 (*16*). To know if this sulfate ion binds to the similar binding site to that for the diphosphate moiety of the allylic substrate FPP, we examined the inhibitory effect of sulfate ion and of inorganic pyrophosphate (PPi). Figure 2 shows the inhibitory effects of both ions on the UPP synthase. As shown in Figure 2a, sulfate ion is a competitive inhibitor with respect to FPP although the K_i value (17 mM) is larger than those of PPi (6 mM) or FP (0.53 mM), both of which have similar structural parts of FPP in their molecular structure. These results suggest that the structural P-loop motif (*16*, *17*) is for the recognition of the diphosphate moiety of the allylic substrate.

Preparation of Site-Directed Mutants of the UPP Synthase. To elucidate the roles of the structural P-loop motif and the disordered region, both of which seem to be important for allylic substrate binding, site-directed mutagenesis studies were carried out with respect to the conserved amino acids in Regions I and III of *M. luteus* B-P 26 UPP synthase. With respect to the structural P-loop motif, Gly-32 was changed to Arg (G32R), and Arg-33 was replaced with Ala (R33A). Moreover, Arg-42, which is complementarily conserved with respect to Gly-32 and occupies the space for the Gly (*16*), was changed to Gly (R42G). The double-mutated enzyme G32R-R42G was also prepared. To construct expression systems for these mutants, oligonucleotide-mediated mutagenesis was conducted into plasmid pMluUEX, which carries the wild-type UPP synthase gene (*18*). After transformation of *E. coli* BL21 (DE3) with each of the expression plasmids, every UPP synthase mutant was overproduced and purified. All the mutated enzymes showed similar chromatographic properties to those of the wild-type UPP synthase during the purification procedures (*9*).

Kinetic Analysis of UPP Synthase Mutants. Kinetic constants for the allylic substrate FPP or *Z*-GGPP and the homoallylic substrate IPP are listed in Table 1. Substitutions of Asp-29 or Arg-80 with Ala resulted in 100- and 400-fold lower k_{cat} values than that of the wild type, respectively, suggesting both of the conserved amino acid residues are essential for the catalytic function. These results were consistent with those reported by Pan et al., in which the D26A or R77A mutant (analogous residue to D29 or R80 in the *M. luteus* B-P 26 enzyme) UPP synthase from *E. coli* showed marked decrease in k_{cat} values (*29*). On the other hand, compared to the wild-type enzyme, R33A showed a 40-fold increase in the K_m value for FPP, with 30-fold decreased k_{cat} values, indicating that Arg-33 in the structural P-loop is important for the binding of FPP.

Among the four residues in the structural P-loop motif of the UPP synthase, Gly-32 is not conserved strictly but is alternatively located with Arg-42, which is close to Gly-32 in the crystal structure (*16*). The double-mutated enzyme with respect to the two complementarily conserved residues, G32R-R42G showed approximately 60% enzymatic activity to that of the wild-type. However, G32R, which contains

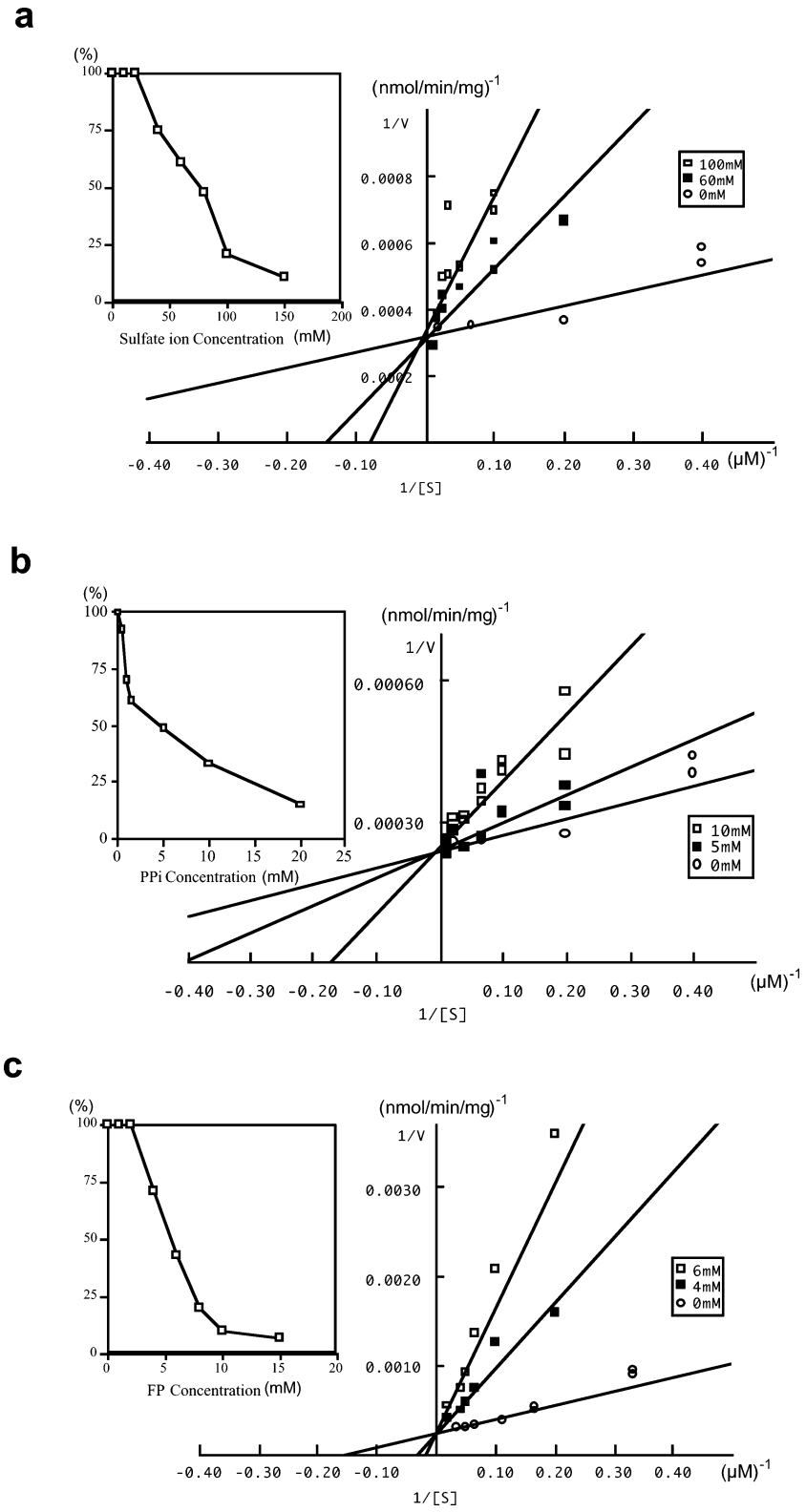

FIGURE 2: Lineweaver−Burk plots of sulfate ion (a), PPi (b), and FP (c) determined at three different inhibitor concentrations on wild-type UPP synthase. (a: 0 (○), 60 (■), or 100 mM (□); b: 0 (○), 5 (■), or 10 mM (□); and c: 0 (○), 4 (■), or 6 mM (□)). Each inset shows inhibitory effect.

Arg residues at both positions of 32 and 42, showed 4-fold decreased k_{cat} value, whereas another mutant R42G, which lacks Arg in both positions, showed an 8-fold decreased k_{cat} value. These results suggest the possible complementation between the corresponding positions of 32 and 42 among the *cis*-prenyltransferases.

To our surprise, most of all mutants showed comparable K_m values for *Z*-GGPP, which is the first intermediate in the consecutive condensation of IPP to the natural substrate FPP, to that of the wild-type enzyme. Moreover, the K_m value for IPP of R33A was calculated to be 60-fold larger than that of wild type, although the precise value of which could not

Table 1: Kinetic Parameters of the Wild-Type and Mutant UPP Synthase of *M. luteus* B-P 26

enzyme	K_m (FPP) (μM)	K_m (*Z*-GGPP) (μM)	K_m (IPP) (μM)[a]	$k_{cat} \times 10^{-3}$ (FPP) (s^{-1})[b]	$k_{cat} \times 10^{-3}$ (*Z*-GGPP) (s^{-1})[b]
wild-Type	3.6 ± 1.6 (8.8 ± 3.0)[d]	8.2 ± 4.0	7.8 ± 2.8	1660 ± 150	1770 ± 250
D29A	33.2 ± 7.0	5.0 ± 0.7	45.2 ± 19	16.7 ± 1.2	25.8 ± 0.8
R33A	144 ± 24[c] (64.0 ± 18)[c,d]	9.5 ± 3.8[c]	>500	54.2 ± 3.1	81.3 ± 6.6
E76Q	2.3 ± 0.5	2.3 ± 0.2	11.4 ± 1.7	505 ± 20	604 ± 11
R80A	23.1 ± 6.1	6.1 ± 1.0	49.1 ± 8.9	4.00 ± 0.3	5.79 ± 0.97
E84Q	11.4 ± 1.2	6.0 ± 1.1	30.7 ± 2.4	1090 ± 55	1070 ± 109
G32R	3.3 ± 0.2	4.3 ± 0.4	6.0 ± 1.5	462 ± 8.6	608 ± 16
R42G	4.9 ± 0.8	4.5 ± 0.7	24.2 ± 6.6	216 ± 11	382 ± 16
G32R-R42G	5.0 ± 1.3	5.9 ± 2.8	5.9 ± 0.9	960 ± 62	1110 ± 166

[a] For the reaction with FPP as the allylic substrate. [b] The k_{cat} values were calucated by the V_{max} values for the reaction with FPP. [c] [4-^{14}C] IPP concentration was kept at 500 μM. [d] The K_m value for E-GGPP.

be obtained because of the limit of the solubility of FPP. These results suggest that the binding features of the two allylic substrates, FPP and *Z*-GGPP, are distinctly different than each other.

Both of the mutants of the conserved Glu's at positions 76 and 84, which are in the undefined region by the X-ray analysis (*16*), showed no significant changes in the kinetic constants except for the moderate changes in the k_{cat} values. Similar results have been reported in a previous paper (*19*); the *E. coli* mutant enzyme E73A or E81A gave decreased k_{cat} values.

Product Analysis. After enzymatic reaction of the mutant or wild-type enzymes with [4-^{14}C]IPP and FPP as substrates, the radioactive prenyl diphosphate products were hydrolyzed to the corresponding alcohols. Figure 3 shows the results of the reversed phase TLC analyses of the products.

Among the mutant enzymes, only R33A gave a different product distribution pattern (Figure 3a, lane 3). Because of the reduced enzymatic activity, it was necessary to increase both the enzyme concentrations and the incubation times to obtain a reliable amount of the reaction products of D29A, R33A, and R80A (Figure 3a, lanes 2, 3, and 5, respectively). All the mutant enzymes except for R33A were found to catalyze the formation of UPP as the major product as well as some intermediates having shorter prenyl chains, which is moderately similar to the reaction catalyzed by the wild-type enzyme. However, the product distribution of the R33A mutant resulted in a resembling pattern to that of the wild type when the reaction of R33A was carried out for a prolonged incubation period (Figure 3b) or with a larger amount of enzyme (Figure 3c), although the major product chain length became C_{50}, and production of C_{40} and C_{45} intermediates were distinct. It is noteworthy that the double-mutated enzyme G32R-R42G showed a product distribution pattern exactly similar to that of the wild type, which gives a detectable amount of C_{60} product.

Dissociation Constants for the Substrates. A membrane retention assay was carried out to determine the dissociation constants (K_d) of the UPP synthase for FPP and for IPP. The K_d for FPP was determined to be 83 ± 13 nM. On the other hand, the K_d for the homoallylic substrate IPP was not obtained in the absence of the allylic substrate. In the presence of FP, which is a competitive inhibitor for the allylic substrate FPP as shown in the above section (Figure 2c), the UPP synthase showed affinity to IPP, and the K_d for IPP was calculated to be a large value of 650 ± 120 nM.

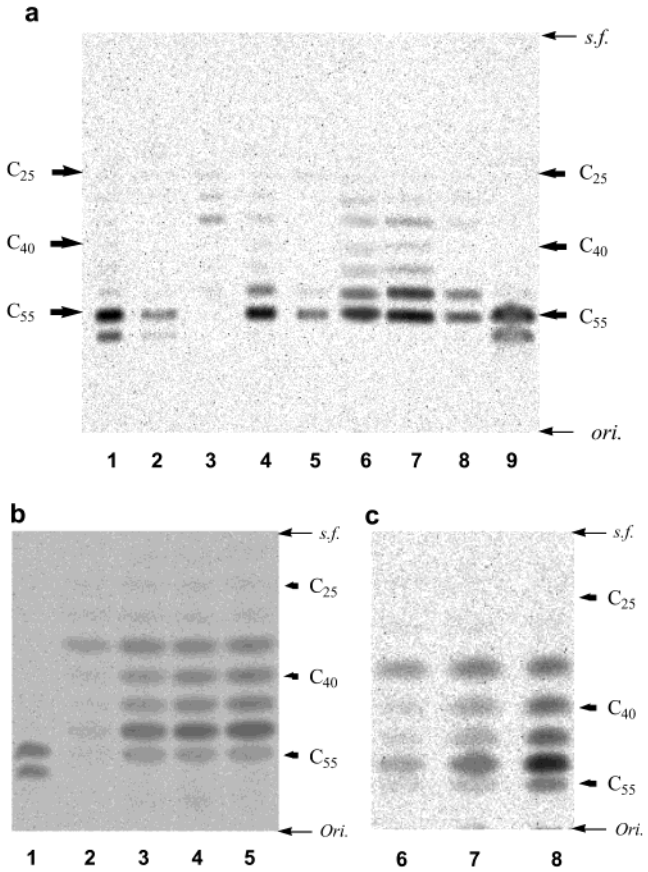

FIGURE 3: Product analysis of wild-type and mutant UPP synthases by reversed-phase TLC. *s.f.*, solvent front; *ori.*, origin. (a) Lane 1, wild-type UPP synthase; lane 2, D29A; lane 3, R33A; lane 4, E76Q; lane 5, R80A; lane 6, E84Q; lane 7, G32R; lane 8, R42G; and lane 9, G32R-R42G. Each reaction mixture containing 100 ng (lanes 1, 4, 6−8) or 2000 ng (lanes 2, 3, 5) of wild-type or mutant enzyme was incubated at 37 °C for 15 min. (b and c) Product distribution of R33A reaction with respect to the incubation periods (b) and the amount of enzyme (c). (b) Lane 1, wild-type UPP synthase, incubation time of 30 min, 100 ng of enzyme; lanes 2−5, incubation time of 60, 120, 240, and 360 min, respectively, with 2000 ng of enzyme; (c) lanes 6−8, 500, 1000, and 2000 ng of enzyme, respectively, with incubation time of 360 min.

DISCUSSION

To elucidate the significance of the conserved amino acid residues in Regions I and III of *M. luteus* B-P 26 UPP synthase, several kinds of site-specific mutant enzymes were

prepared, and their changes in catalytic function were analyzed.

According to the kinetic results, we propose a hypothetical binding site for the allylic substrate FPP of the UPP synthase in more detail than that in our previous reports (*16*, *18*). Arg-33 in the structural P-loop uses its charged side chain to bind directly with the diphosphate moiety of FPP. The increased production of C_{40} and C_{45} intermediates by R33A after prolonged incubation time or with larger amount of the mutant enzyme may reflect the extremely decreased affinity of the structural P-loop for FPP, affecting the termination mechanism of the prenyl chain elongation. Asp-29 as well as Arg-80 are essential for the catalytic function (*16*), probably for the C−O bond cleavage to form an allylic cation to start the prenyltransferase reaction.

According to the crystal structure of the *M. luteus* B-P 26 UPP synthase, Arg-42 is located close to Gly-32, which is in the structural P-loop, interacting with sulfate ion in the X-ray crystal structure without any substrates (*16*). Although G32R-R42G showed a 1.6-fold lower k_{cat} value than that of wild type, this double mutant produces C_{55} and C_{60} products in a similar ratio as that of wild type (Figure 3a). This result shows that the catalytic function was influenced slightly by the double mutation. On the other hand, R42G, which does not have Arg at both positions, showed an 8-fold lower k_{cat} value, and G32R, which has Arg's at both positions, showed a 4-fold decreased k_{cat} value. The two Arg residues in the G32R mutant seem to have some repulsion with each other, so that Arg-42 may not be located at a proper position. Taken together, these results support directly the complement of the Arg-42 residue by that of the position at Gly-32 in the structural P-loop in some of the cis-type prenyltransferases such as that from *Caenorhabditis elegans*, which have Arg at the corresponding position in the structural P-loop. Figure 4 shows the proposed structural relationships with respect to the complementarily conserved Arg-42.

In our filter assay experiments, the K_d value for IPP of the UPP synthase is larger than that for FPP. Furthermore, IPP can only bind with the UPP synthase in the presence of the allylic substrate FPP. This result distinctly suggests that the order of the substrate binding in the consecutive condensation reaction to that of the allylic substrate FPP binds first, and then IPP binds. The earlier pre-steady-state kinetic analysis reported by Pan et al. suggested that the IPP condensation is the rate-limiting step (*30*). Thus, it is reasonable to assume that those mutants having large K_m values for FPP will show larger K_m values for IPP because lowered affinity to FPP may affect the binding of IPP to the enzyme. If the substitution of amino acid residues affected the FPP binding, K_m values not only for FPP but also for IPP must be increased. In fact, mutant enzymes R33A, D29A, and R80A, which showed 6−40-fold increases in the K_m values for FPP, also gave larger K_m values for IPP. Especially in the case of R33A, both K_m values for FPP and for IPP resulted in 40- and more than 60-fold increases, respectively. Dramatic changes in the binding ability to the allylic substrate may cause a striking effect to the IPP binding site. On the other hand, those mutant enzymes that have some changes in the IPP binding sites such as F73A and S74A have been shown to give no change in the K_m values for allylic substrate (*24*). Similar results have been reported in our previous paper (*18*). The dissociation constant for FPP is much smaller than

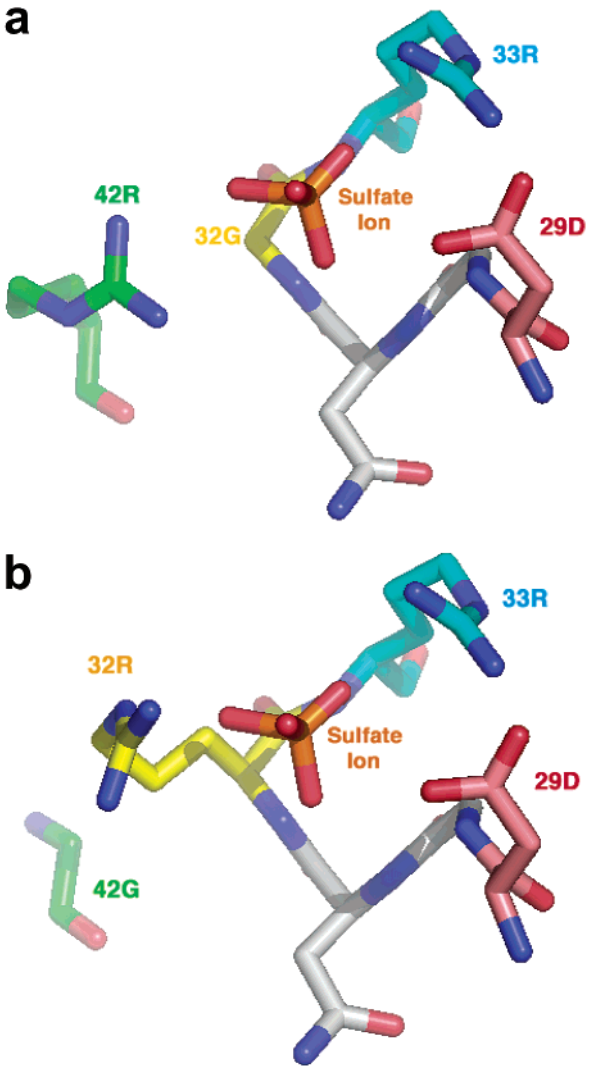

FIGURE 4: Hypothetical model about the complementarily conserved residues, Gly-32 and Arg-42. (a) Wild-type; in the structural P-loop motif Gly-32 is located close to Arg-42, which is essential for the catalytic function. (b) G32R-R42G; the Gly-32 is complementarily conserved with Arg-42, which can be exchanged with each other. These figures were prepared with the software Swiss-PDB Viewer (*34*) and PyMOL (*35*).

the K_d for a fluorescent analogue of FPP with *E. coli* UPP synthase (*31*). This may be attributable to the reason that the analogue is not a natural substrate, as the K_d values for FPP with respect to the yeast protein farnesyltransferase (*32*) or to the *Bacillus subtilis* heptaprenyl diphosphate synthase (*33*) have been reported to be within a similar order, 75 and 60 nM, respectively, both of which are considerably lower than the K_m values for the corresponding enzymes.

It is noteworthy that all mutants determined in this study showed K_m values for *Z*-GGPP comparable to that of the wild-type enzyme. On the other hand, as shown in Table 1, the mutant R33A showed 8-fold increased K_m values for the trans-type allylic substrate (all-*E*)-GGPP. These results suggest that the substitution of Arg-33 affected the binding of (all-*E*)-GGPP substrate as well as FPP but not for the geometric isomer, *Z*-GGPP. This fact implies that the binding site for the intermediate *Z*-GGPP, which has a newly formed cis-double bond, is different to that for the FPP or (all-*E*)-GGPP, whose diphosphate moiety will bind to the structural P-loop motif. When the first cis-type prenyl chain elongation

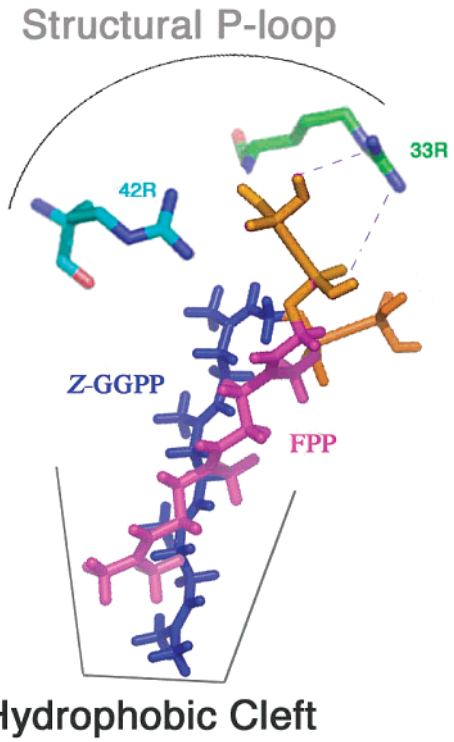

FIGURE 5: Hypothetical allylic substrate, FPP and *Z*-GGPP binding model of UPP synthase of *M. luteus* B-P 26. If the prenyl chain of *Z*-GGPP, which has a *cis*-isoprene unit, binds to the similar region for that of FPP in the large hydrophobic cleft after the relocation by keeping the C-1 position in the similar proximity to the 4′-position of IPP, the diphosphate moiety colored in orange cannot bind to the structural P-loop. This figure was prepared with software, PyMOL (*35*).

occurs between one molecule of IPP and (all-*E*)-FPP or (all-*E*)-GGPP that bound to the structural P-loop motif, the newly formed intermediate having a cis-double bond in the α-isoprene unit has to relocate to a new allylic substrate binding site for the next IPP condensation with a *cis*-allylic substrate (Figure 5). The following cis-type prenyl chain elongation may occur with the intermediates having a *cis*-isoprene unit by consecutive relocation to the new binding site for allylic diphosphates having a *cis*-isoprene unit. This hypothesis may imply the existence of a second domain for IPP binding and catalysis to further elongate the *cis*-prenyl chain. However, as illustrated in Figure 5, if the C-1 position of the intermediate *cis*-prenyl diphosphate can be located in the similar proximity to the 4′-position of IPP, which is bound to the original IPP binding site, further elongation by forming a C−C bond between *cis*-allylic diphosphate and IPP might be possible. These results seem to be quite unique and different from those of the *E*-type enzyme, such as FPP synthase. Elucidation of the X-ray crystal structure of the UPP synthase with these intermediate substrates bound would provide a direct feature of a precise reaction mechanism of a cis-type prenyl chain elongating enzyme.

REFERENCES

1. Ogura, K., and Koyama, T. (1998) *Chem. Rev. 98*, 1263−1276.
2. Koyama, T., and Ogura, K. (1999) in *Comprehensive Natural Products Chemistry* (Cane, D. E., Ed.) Vol. 2, pp 69−96, Pergamon/Elsevier Science, Oxford.
3. Marrero, P. F., Poulter, C. D., and Edwards, P. A. (1992) *J. Biol. Chem. 267*, 21873−21878.
4. Joly, A., and Edwards, P. A. (1993) *J. Biol. Chem. 268*, 26983−26989.
5. Song, L., and Poulter, C. D. (1994) *Proc. Natl. Acad. Sci. U.S.A. 91*, 3044−3048.
6. Koyama, T., Tajima, M., Sano, H., Doi, T., Koike-Takeshita, A., Obata, S., Nishino, T., and Ogura, K. (1996) *Biochemistry 35*, 9533−9538.
7. Tarshis, L. C., Yan, M., Poulter, C. D., and Sacchettini, J. C. (1994) *Biochemistry 33*, 10871−10879.
8. Tarshis, L. C., Proteau, P. J., Kellogg, B. A., Sacchettini, J. C., and Poulter, C. D. (1996) *Proc. Natl. Acad. Sci. U.S.A. 93*, 15018−15023.
9. Shimizu, N., Koyama, T., and Ogura, K. (1998) *J. Biol. Chem. 273*, 19476−19481.
10. Apfel, C. M., Takacs, B., Fountoulakis, M., Stieger, M., and Keck, W. (1999) *J. Bacteriol. 181*, 483−492.
11. Sato, M., Sato, K., Nishikawa, S., Hirata, A., Kato, J., and Nakano, A. (1999) *Mol. Cell. Biol. 19*, 71−83.
12. Schulbach, M. C., Brennan, P. J., and Crick, D. C. (2000) *J. Biol. Chem. 275*, 22876−22881.
13. Oh, S. K., Han, K. H., Ryu, S. B., and Kang, H. (2000) *J. Biol. Chem. 275*, 18482−18488.
14. Kobayashi, M., Ito, M., Koyama, T., and Ogura, K. (1985) *J. Am. Chem. Soc. 107*, 4588−4589.
15. Ito, M., Kobayashi, M., Koyama, T., and Ogura, K. (1987) *Biochemistry, 26*, 4745−4750.
16. Fujihashi, M., Zhang, Y.-W., Higuchi, Y., Li, X.-Y., Koyama, T., and Miki, K. (2001) *Proc. Natl. Acad. Sci. U.S.A. 98*, 4337−4342.
17. Kinoshita, K., Sadanami, K., Kidera, A., and Go, N. (1999) *Protein Eng. 12*, 11−14.
18. Fujikura, K., Zhang, Y.-W., Yoshizaki, H., Nishino, T., and Koyama, T. (2000) *J. Biochem. (Tokyo) 128*, 917−922.
19. Ko, T. P., Chen, Y. K., Robinson, H., Tsai, P. C., Gao, Y. G., Chen, A. P., Wang, A. H., and Liang, P. H. (2001) *J. Biol. Chem. 276*, 47474−47482.
20. Davisson, V. J., Woodside, A. B., Neal, T. R., Stremler, K. E., Muehlbacher, M., and Poulter, C. D. (1986) *J. Org. Chem. 51*, 4768−4779.
21. Inoue, H., Korenaga, T., Sagami, H., Koyama, T., and Ogura, K. (1994) *Biochem. Biophys. Res. Commun. 200*, 1036−1041.
22. Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual*, 2nd ed., Cold Spring Harbor Laboratory, Cold Spring Harbor, New York.
23. Sanger, F., Nicklen, S., and Coulson, A. R. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 5463−5467.
24. Kharel, Y., Zhang, Y.-W., Fujihashi, M., Miki, K., and Koyama, T. (2001) *J. Biol. Chem. 276*, 28459−28464.
25. Bradford, M. A. (1976) *Anal. Biochem. 72*, 248−254.
26. Koyama, T., Fujii, H., and Ogura, K. (1985) *Methods Enzymol. 110*, 153−155.
27. Dolence, J. M., Cassidy, P. B., Mathis, J. R., and Poulter, C. D. (1995) *Biochemistry 34*, 16687−16694.
28. Voet, D., and Voet, J. G. (1995) in *Biochemistry*, 2nd ed., Vol. II, pp 1053−1054, John Wiley and Sons, Tokyo-Kagaku Dojin, Tokyo.
29. Pan, J. J., Yang, L. W., and Liang, P. H. (2000) *Biochemistry 39*, 13856−13861.
30. Pan, J. J., Chiou, S. T., and Liang, P. H. (2000) *Biochemistry 39*, 10936−10942.
31. Chen, A. P., Chen, Y. H., Liu, H. P., Li, Y. C., Chen, C. T., and Liang, P. H. (2002) *J. Am. Chem. Soc. 124*, 15217−15224.
32. Dolence, J. M., Cassidy, P. B., Mathis, J. R., and Poulter, C. D. (1995) *Biochemistry 34*, 16687−16694.
33. Zhang, Y.-W., Li, X.-Y., Sugawara, H., and Koyama, T. (1999) *Biochemistry 38*, 14638−14643.
34. Guex, N., and Peitsch, M. C. (1997) *Electrophoresis 18*, 2714−2723.
35. Derano, W. L. (2002) *The Pymol Molecular Graphics System*, DeLano Scientific, San Carlos, CA, http://www.pymol.org

BI027236V