J. Biochem. **133**, 83–91 (2003)
DOI: 10.1093/jb/mvg002

Interconversion of the Product Specificity of Type I Eubacterial Farnesyl Diphosphate Synthase and Geranylgeranyl Diphosphate Synthase through One Amino Acid Substitution

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Received August 8, 2002; accepted October 26, 2002

Prenyltransferases catalyze the sequential condensation of isopentenyl diphosphate into prenyl diphosphates with specific chain lengths. Pioneering studies demonstrated that the product specificities of type I prenyltransferases were mainly determined by the amino acid residues at the 4th and 5th positions before the first aspartate-rich motif (FARM) of the prenyltransferases. We previously cloned a type I geranylgeranyl diphosphate synthase (GGDPSase) gene from *Streptomyces griseolosporeus* MF730-N6 [Hamano, Y., Dairi, T., Yamamoto, M., Kawasaki, T., Kaneda, K., Kuzuyama, T., Itoh, N., and Seto, H. (2001) *Biosci. Biotechnol. Biochem.* 65, 1627–1635]. In this study, a prenyltransferase gene was cloned from *Streptomyces argenteolus* A-2 and was confirmed to encode a type I farnesyl diphosphate synthase (FDPSase). Interestingly, the amino acid residues at the 4th and 5th positions before the FARM were the same in these two enzymes. To identify the amino acid that determines the product chain length, mutated enzymes, GGDPSase (L-50S), FDPSase (S-50L), GGDPSase (V-8A), FDPSase (A-8V), GGDPSase (A+57L), and FDPSase (L+58A), in which the amino acid residue at the –50th, –8th, and +57th (58th) position before or after the FARM was substituted with the corresponding amino acid of the other enzyme, were constructed. The GGDPSase (A+57L) and FDPSase (L+58A) produced farnesyl diphosphate and geranylgeranyl diphosphate, respectively. On the other hand, the other mutated enzymes produced prenyl diphosphates with the same chain lengths as the wild type enzymes did. These results showed that the amino acid residue at the 57th (58th) position after the FARM also played an important role in determination of the product specificity.

Key words: farnesyl diphosphate synthase, geranylgeranyl diphosphate synthase, product specificity, site-directed mutagenesis, *Streptomyces*.

Abbreviations: DMAPP, dimethylallyl diphosphate; IPP, isopentenyl diphosphate; GDP, (*E*)-geranyl diphosphate; FDP, (*E*,*E*)-farnesyl diphosphate; FDPSase, farnesyl diphosphate synthase; GGDP, (*E*,*E*,*E*)-geranylgeranyl diphosphate; GGDPSase, geranylgeranyl diphosphate synthase; ORF, open reading frame.

Prenyltransferases catalyze the sequential condensation of isopentenyl diphosphate (IPP) with allylic diphosphates such as dimethylallyl diphosphate (DMAPP) into linear prenyl diphosphates with specific chain lengths (*1*). In many cases, these prenyl diphosphates undergo a range of cyclizations to produce the parent skeletons of isoprenoid compounds, followed by a variety of modifications yielding many thousands of different isoprenoid metabolites (*2*). The prenyltransferases are classified into two groups with respect to the stereochemistry of the *E* or *Z* double bond that is formed through the condensation. Until now, many enzymes that form an (all-*E*)-prenyl diphosphate have been isolated from various organisms (*1*). On the other hand, a gene encoding an enzyme that yields an (all-*Z*)-prenyl diphosphate was recently cloned from *Mycrococcus luteus* B-P 26 (*3*).

A number of genes encoding farnesyl diphosphate synthases (FDPSases), which are *E*-prenyl diphosphate synthases, have been cloned and characterized (*1*). The FDPSases were classified into two groups based on the amino acid sequence of the first aspartate-rich motif (FARM), which is known to be a substrate binding site. Type I FDPSases contain a DDXXD motif, and are usually found in eukaryotes and archaebacteria. On the other hand, type II FDPSases contain a DDXXXXD motif, and generally exist in eubacteria (*4*). On systematic analyses, these enzymes appeared to have evolutionally originated from an ancestral archaeal geranylgeranyl diphosphate synthase (GGDPSase), and were divided into two types, types I and II (*4*).

Although GGDPSases and FDPSases exhibit high amino acid identity with each other, different numbers of IPPs are incorporated into the final products catalyzed

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by them. The product specificities of these enzymes have been extensively investigated and were revealed to be determined by amino acid residues around FARM. In type I FDPSases, the amino acid residues at the 4th, 5th, 8th, and/or 11th positions before the FARM were shown to determine the product specificity (*4*–*7*). Bulky amino acids at these positions were proposed to block the elongation of the product, which results in the formation of a prenyl diphosphate with a short chain length. On the other hand, the product specificity of type II FDPSases were revealed to be determined by an aromatic amino acid at the 5th position before the FARM, two amino acids inserted into the FARM, and so on (*4*).

We have been interested in the isoprenoid cyclases of actinomycetes. We previously cloned two adjacent gene clusters containing mevalonate pathway genes and terpentecin (TP) biosynthetic genes from *Streptomyces griseolosporeus* MF730-N6, a diterpene antibiotic-TP producer (*8*–*10*). In the former cluster, seven genes encoding GGDPSase, mevalonate kinase, mevalonate diphosphate decarboxylase, phosphomevalonate kinase, isopentenyl diphosphate isomerase, HMG-CoA reductase, and HMG-CoA synthase, respectively, were suggested to exist in that order (*8*). In the latter cluster, the genes for two diterpene cyclases, which were essential for the conversion of GGDP into terpentetriene that had the same basic skeleton as terpentecin, were found (*9*, *10*). We are now trying to clone a tetraterpene cyclase gene from *Streptomyces argenteolus* A-2, a tetraterpene antibiotic-KS-505a producer (*11*). In this study, we have tried to clone a gene encoding an octaprenyl diphosphate synthase that supplies a substrate for the tetraterpene cyclase from the KS-505a producer to determine if the tetraterpene cyclase gene might be clustered with the octaprenyl diphosphate synthase gene in a manner similar to in the TP producer. One putative prenyltransferase gene was successfully cloned from the strain. The gene was confirmed to encode a FDPSase through characterization with a recombinant enzyme.

Although we could not clone an octaprenyl diphosphate synthase gene, we noticed that the FDPSase has a very similar primary structure to that of the GGDPSase cloned from *S. griseolosporeus* MF730-N6 (50% identity), and that the amino acid residues at the 4th, 5th, 8th, and 11th positions before the FARM of these two enzymes were also the same except for that at the 8th position. Moreover, the amino acids at the 8th position before the FARM of the GGDPSase and FDPSase were Val and Ala, suggesting that the above-mentioned hypothesis as to the product specificities of prenyltransferases can not be applied in this case. Therefore, the mechanism underlying chain termination in these two enzymes was investigated by constructing mutated enzymes, in which amino acid residues before or after the FARM were substituted with the corresponding amino acids of the other enzyme, and the chain lengths of the final products were analyzed. Kinetic studies on these mutated enzymes are also reported.

EXPERIMENTAL PROCEDURES

Chemicals—[α-^{32}P]dCTP and [1-^{14}C]IPP (CFA476, 2.15GBq/mmol) were obtained from Amersham Pharmacia. IPP, DMAPP, GDP, FDP, and GGDP were purchased from Sigma-Aldrich. An other chemicals were of analytical grade.

Bacterial Strains and Culture Conditions—*Streptomyces argenteolus* A-2 was used as a source of total DNA for the cloning experiment. The media and growth conditions for the strain were as described by Nakanishi *et al*. (*11*). *E. coli* JM110 {*rpsL thr leu thi lacY galK ara tonA tsx dam dcm supE44* /F′ [*traD proAB lacI*^{q} *lacZ*ΔM15]} (Toyobo) and a cosmid pWE15 (Toyobo) were used for the preparation of a genomic library. *E. coli* JM110 and plasmids, pUC118 and pUC119, were used for sequencing analysis. *E. coli* BL21 (DE3) [F^{-} *ompT hsdS*$_{B}$ (*r*$_{B}^{-}$ *m*$_{B-}^{-}$) *gal dcm* (DE3)] and a plasmid, pET21b (Novagen), were used for expression of the C-terminal His-tagged fusion proteins.

DNA Isolation and Manipulation—Plasmids from *E. coli* were prepared using a Qiagen plasmid kit. All restriction enzymes, T4 ligase, and calf intestinal alkaline phosphatase were obtained from Toyobo, and used according to the manufacturer's instructions. Transformation of *E. coli* with plasmid DNA by electroporation was performed under standard conditions using a BTX ECM 600 electroporation system (Biotechnologies and Experimental Research). The procedure for cosmid library construction was previously described (*12*). Other general procedures were performed as described by Maniatis *et al.* (*13*).

Cloning of a Prenyltransferase Gene from S. argenteolus—Degenerate primers with an additional restriction site (underlined), 5′-GACGAATTCGC(C/G)ATC(C/T)T(C/G)GC(C/G)GG(C/G)GAC(C/G/T)T(C/G)(C/T)T-3′ and 5′-ACCAAGCTT-GTCGTC(C/G)AC(C/G)A(C/G/T)CTGGAA(C/G)(A/G)(A/C/G)(C/G)A-3′, corresponding to highly conserved amino acid sequences, AILAGDIVL and AFQIVDDIKL, respectively, were constructed. PCR amplification was performed with a Takara LA PCR kit (Takara Shuzo, Kyoto, Japan) under the following conditions: denaturation at 94°C for 1 min, annealing at 50°C for 1.5 min, and elongation at 72°C for 1 min, 30 cycles. The amplified 0.4-kb fragment was digested with *Hin*dIII and *Eco*RI, followed by purification, and then inserted into the same site of pUC118. After DNA sequencing, a cosmid library of *S. argenteolus* A-2 was screened by means of colony hybridization with the ^{32}P-labeled 0.4-kb amplified fragment as a probe. A positive cosmid clone was selected, and a 5.6-kb *Bam*HI fragment, which again hybridized to the probe on Southern blot analysis, was subcloned into the same sites of pUC119. After construction of a series of plasmids, sequencing was performed with an automatic DNA sequencer (model 4000L; Li-Cor).

Overproduction of the GGDPSase and FDPSase in E. coli—To obtain the entire gene without an excessive flanking region, PCR amplification was carried out. The PCR conditions were the same as those described above. (i) Amplification of the GGDPSase gene; the 5′ and 3′ primer with an additional restriction site (underlined) had the respective sequences, 5′-GGAATTCCATATGTA-CACCGACACCGCCGA-3′ and 5′-ACCAAGCTTGTGGT-TCCTGAAA GCGACATA-3′, which were designed on the basis of the nucleotide sequence of the GGDPSase gene. The amplified PCR product was digested with *Eco*RI and *Hin*dIII, separated by agarose gel electrophoresis, and

then purified with a Gel Extraction Kit (Qiagen). The fragment was inserted into the same site of pUC118, and the nucleotide sequence of the whole-amplified gene was confirmed to have undergone no error matching during the PCR by sequencing of both strands. After sequence confirmation, the *Nde*I–*Hin*dIII fragment was inserted into the same site of C-terminal His-Tagged fusion vector pET21b. In the resulting plasmid, pGGDPS, a recombinant protein was expressed under the control of the T7 promoter. (ii) Amplification of the FDPSase gene; the 5′ primer, 5′-GGAATTCCATATGACGGCGATCGAGGTCAC-3′, and 3′ primer, 5′-ACCAAGCTTGT GGTTCCTGAAAG-CGACATA-3′, were used for PCR amplification. Other procedures were the same as those for the construction of pGGDPS. The plasmid constructed was designated as pFDPS.

Construction of Mutated Prenyltransferases — Site-directed mutagenesis was carried out with a Mutan-Super Express Km kit (Takara Shuzo, Kyoto). The oligonucleotides used were as follows: GGDPSase (L-50S), 5′-GCC-CCGCCAGGTCCGACCAGCCGAAGTGGT-3′; FDPSase (S-50L), 5′-CGCCTCGCTCGTCCAGCCAGCCGAA GTGGT-3′; GGDPSase (V-8A), 5′-GGGAGAAGTTGTGCGCCAACT-CGACCGCGA-3′; FDPSase (A-8V), 5′-GGGAG AAATTG-TGCACCAGCTCGACCGCGA-3′; GGDPSase (A+57L), 5′-CCATCTGCCCTCGAGGAGCGTCGCGATCG-3′; FDPSase (L+58A), 5′-CGTTCTGCCCCTCCGCCAGTTCCTGGATGG-3′; GGDPSase (S-4F), 5′-CGTCGTGGAGCAGGAAGAAGT-TGTGCACCA-3′; and FDPSase (F-5A), 5′-CGTGCAGGAG-GGAGGCATTGTGCGCCAGCT-3′. The mutated codons are underlined. After all the mutations had been confirmed by DNA sequencing, the *Nde*I–*Hin*dIII fragment was inserted into pET21b. Plasmids, pGGDPS (L-50S), pFDPS (S-50L), pGGDPS (V-8A), pFDPS (A-8V), pGGDPS (A+57L), pFDPS (L+58A), pGGDPS (S-4F), and pFDPS (F-5A), in which recombinant proteins were expressed as C-terminal 6× His-tagged fusion proteins, were selected.

E. coli BL21 (DE3) harboring the recombinant plasmid was grown at 37°C in Luria Broth medium in the presence of ampicillin (100 μg/ml). Expression of the recombinant protein was induced by adding 0.1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) when the optical density at 600 nm reached about 0.8. The cultivation was continued for an additional 15 hr at 18°C. Purification of the His-tagged recombinant proteins was performed according to the manufacturer's instructions (Qiagen). The purified proteins were analyzed by SDS-PAGE on 10% gels.

Enzyme Assay—An enzyme assay to determine the product chain length was conducted as follows. The reaction mixture comprised 50 mM TES Buffer (pH 8.0), 5 mM 2-mercaptoethanol, 5 mM $MgCl_2$, 50 μM allylic substrate, 50 μM [1-^{14}C]IPP, 20% glycerol, 0.1% Triton X-100, and 20 μg/ml of the purified recombinant enzyme. This reaction mixture was incubated at 30°C for 1 h. After the product had been extracted three times with 1-butanol, the diphosphate of the product was hydrolyzed with an acid phosphatase by the method described by Koyama *et al*. (*14*). The resulting hydrolysates were analyzed by TLC according to the method of Ohnuma *et al*. (*7*). For the kinetic studies, radiometric assays were performed by the same method as that described by Sagami *et al*. (*15*).

RESULTS

Cloning of a Prenyltransferase Gene from S. argenteolus— So far, many deduced amino acid sequences of prenyltransferases have been reported (*1*). Comparison of the primary structures has revealed that several domains are conserved in these enzymes (*1*). Therefore, we tried to clone an octaprenyl diphosphate synthase gene by PCR with primers that were designed based on the conserved amino acid sequences. A 0.4-kb fragment was amplified by PCR under the conditions given under "EXPERIMENTAL PROCEDURES." A cosmid clone was selected by colony hybridization with the fragment as a probe, which had been confirmed to carry a putative prenyltransferase gene by DNA sequencing, and subjected to Southern blot analysis. A 5.8-kb *Bam*HI fragment, which again hybridized to the probe, was prepared from the cosmid, and the nucleotide sequence of the fragment was determined. Finally, four complete ORFs (ORF2-5) and one truncated ORF (ORF1) were suggested to exist in the fragment. To determine the function of each of the ORFs deduced on DNA sequencing, we searched databases with their translated products by means of sequence similarity search programs BLAST (*16*) and FASTA (*17*). In brief, the truncated ORF1, ORF2, ORF3, ORF4, and ORF5 exhibited significant similarity to a protein essential for sporulation of *Streptomyces griseus* {DNA Data Bank of Japan (dad): L76204–1, 73% identity}, a putative prenyltransferase of *Streptomyces coelicolor* A3(*2*) (AL391763–12, 57%), a putative ATP-binding protein of *S. coelicolor* A3(*2*) (AL035636–23, 60%), a probable glutaryl-CoA dehydrogenase of *Deinococcus radiodurans* strain RI (pir: A75505, 53%), and a hypothetical protein of *S. coelicolor* A3(*2*) (dad: AL1334659–17, 47%), respectively.

Product Specificity of the Prenyltransferase—To examine the product specificity of the ORF2 product, a recombinant ORF2 was expressed in *E. coli* as a C-terminal His-tagged fusion protein, and then an *in vitro* assay was performed. Soluble protein extracts of *E. coli* cells harboring pFDPS and pET21b (no insert) were analyzed by SDS-PAGE. The His-tagged ORF2 with a molecular mass of about 36 kDa, which was in good agreement with the value calculated from the deduced amino acid sequences of the enzymes, was expressed at a high level. The expressed proteins were then purified (Fig. 1) and used for the enzyme assay. When the assay was carried out with DMAPP and [1-^{14}C]IPP as substrates, FDP was detected as a major product on TLC analysis (Fig. 2A, lane 1). A similar product distribution was observed for a reaction with GDP as the allylic substrate (Fig. 2B, lane 1), and no GGDP or octaprenyl diphosphate was detected with FDP as the allylic substrate (Fig. 2C, lane 1), showing that the prenyltransferase gene cloned in this study encodes an FDPSase.

Amino Acid Sequences before the FARM of the FDPSase and GGDPSase—Considering that the prenyltransferase was confirmed to be a FDPSase and that the other ORF products were suggested to have no relation with those for KS-505a biosynthesis, the tetraterpene cyclase gene was suggested not to form a cluster with that for the cloned FDPSase. However, we noticed the following inter-

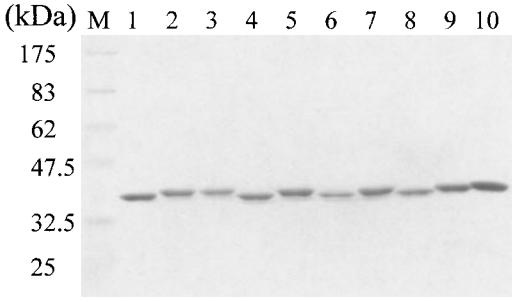

Fig. 1. **Electrophoresis of the overproduced and purified prenyltransferases.** Purified prenyltransferases were analyzed by SDS-PAGE (10%). Proteins were stained with Coomassie Brilliant Blue R-250. M, molecular mass markers; lane 1, purified His-tagged FDPSase; lane 2, purified His-tagged GGDPSase; lane 3, purified His-tagged GGDPSase (V-8A); lane 4, purified His-tagged FDPSase (A-8V); lane 5, purified His-tagged GGDPSase (L-50S); lane 6, purified His-tagged FDPSase (S-50L); lane 7, purified His-tagged GGDPSase (A+57L); lane 8, purified His-tagged FDPSase (L+58A); lanes 9, purified His-tagged GGDPSase (S-4F); lane 10, purified His-tagged FDPSase (F-5A).

esting facts. Firstly, the FDPSase exhibits significant amino acid identity with the GGDPSase that was previously cloned from *S. griseolosporeus* MF730-N6 (50% identity, Fig. 3). Secondly, alignment of the FDPSase with the GGDPSase revealed that the amino acid residues at the 4th, 5th, 8th, and/or 11th positions before the FARM were the same except for that of the 8th position before the FARM (Fig. 3). Moreover, the amino acids at this position of the GGDPSase and FDPSase were Val and Ala, suggesting that the above-mentioned hypothesis can not be applied in this case.

Reassaying of the GGDPSase—It has been reported that the product chain length of each enzyme varies with the *in vitro* reaction conditions (*18*, *19*). Therefore, the product formed by the GGDPSase of *S. griseolosporeus* MF730-N6 was again analyzed with the same method and conditions as those for the FDPSase. As shown in Fig. 2 (lane 2), a major product was again identified to be GGDPSase when DMAPP, GDP, or FDP was used as the allylic substrate.

Construction of Mutated Prenyltransferase and Determination of the Product Chain Length—To determine if the amino acid residue at the 8th position before the FARM was responsible for the product chain length for these enzymes, we constructed two mutated enzymes, in which the amino acids at this position were substituted for each other. The mutated enzymes, GGDPSase (V-8A) and FDPSase (A-8V), were overproduced in *E. coli*, followed by purification (Fig. 1), and used for the *in vitro* assay. As shown in Fig. 2 (lanes 3 and 4), the former and latter produced GGDP and FDP, respectively. This suggested that amino acids other than that at the 8th position before the FARM determined the product specificity of these enzymes.

To identify the amino acids that determine the product chain length, we tried to construct chimera enzymes. Both the GGDPSase gene and FDPSase gene have a *Bst*EII restriction enzyme site in the DNA region encoding the FARM (Fig. 3). By using this site, we tried to replace the amino acids before the FARM of the FDPSase (or the GGDPSase) with those of the GGDPSase (or the FDPSase). However, the chimera enzymes always formed inclusion bodies. Therefore, we constructed several recombinant plasmids, such as a N-terminal 6xHis-fused protein and a thioredoxin-fused protein. However, the expressed proteins again formed inclusion bodies even though several attempts to increase the soluble enzyme by decreasing the amount of IPTG added for induction and by prolonging the cultivation at low temperature were made.

Previously, Ohnuma *et al.* succeeded in the conversion of a type II FDPSase of *Bacillus stearothermophilus* into a GGDPSase by random mutagenesis with $NaNO_2$ (*20*).

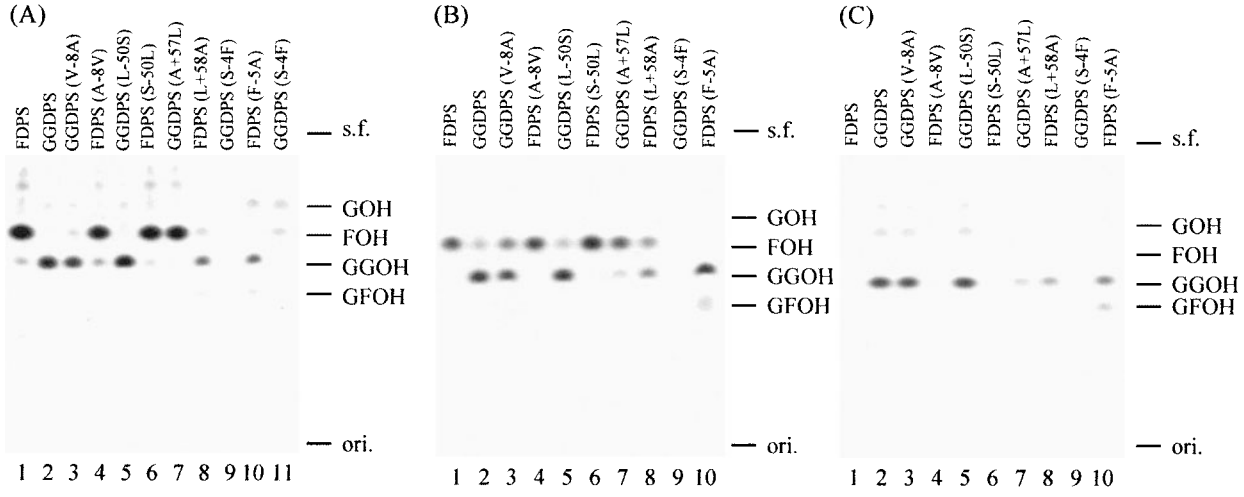

Fig. 2. **TLC autoradiography of the alcohols obtained on hydrolysis of the products formed by the wild and mutated enzymes.** The samples obtained on incubation of [1-^{14}C]IPP and DMAPP (A), GDP (B), or FDP (C) with the enzymes was analyzed by TLC as described under "EXPERIMENTAL PROCEDURES". Numbers indicate the enzymes shown in Fig. 1. Spots of authentic standard alcohols are indicated by arrows: GOH, geraniol; FOH, (all-*E*)-farnesol; GGOH, (all-*E*)-geranylgeraniol; GFOH, (all-*E*)-geranylfarnesol. Ori., origin; sf., solvent front.

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SG        1 MYTDTAETLE SAVDAGRNHT LRAAGIAAPA LRATIDTLGS RIRPIVGYHF GWLDLAGQPT 60
SA          1 MTAIEVT AEGRSAREVL LWSRDTVESA LRPAIDRLPP AMRRIAGYHF GWSDERGEPA 57
                                                                  -50
Baci                1 MAQLSVEQFL NEQKQAVETA LSRYIERLEG PAK------- --LKKAMAYS 41
                                                                  +34
Sulfo                 1 MSYFDNYF NEIVNSVNDI IKSYISGDVP KLY------- ----EASYHL 37
                                :         :    :   *:
                                                          BstEII
                                                           FARM
                                                          ------ -
SG       61 DQGSGKMIRA ALTVLAAEAC GGDVQRAVCG AVAVELVHNF SLLHDDV--M DGDLERRGRP 118
SA       58 RAGGGKAIRP ALVLLAAEAV GGAADAALPA AVAVELAHNF SLLHDDV--M DGDRTRRHRP 115
                                                 -8 -5-4
Baci     42 LEAGGKRIRP LLLLSTVRAL GKDPAVGLPV ACAIEMIHTY SLIHDDLPSM DNDDLRRGKP 101
                                                    +81
Sulfo    38 FTSGGKRLRP LILTISSDLF GGQRERAYYA GAAIEVLHTF TLVHDDI--M DQDNIRRGLP  95
              ::** :*:  :   :     *     :    : *:*: *:: :*:***:  * * *  **  *
                                              +71+74+77        +85

SG      119 TVWATLGVPA AILAGDVLLA RGCAILEGD- SEHRTWATEL -------LTE TIATLAEGQM 170
SA      116 TAWTVFGVSP AILAGDALLA LAFDVLATAG HPAAPEATRI -------FGA AIQELLEGQN 168
                                                                  +57(+58)
Baci    102 TNHKVFGEAM AILAGDGLLT YAFQLITEID DERIPPSVRL RLIERLAKAA GPEGMVAGQA 161
                                                                    +157
Sulfo    96 TVHVKYGLPL AILAGDLLHA KAFQLLTQAL RGLPSETIIK AFD---IFTR SIIIISEGQA 152
             *     * :  ****** * :  :  ::         : :                   :  **
              +99            +114
               +101               +118

SG      171 ADVAFEHRTT ISLEEALAVS EAKTAALLSC ACELGAGLSG GGAEARERLA KFGWHLGMAF 230
SA      169 ADMAFERRQD VELAECLVMA AGKTGALLGC ACALGALLGG GAPEQVEHLR AFGRDLGLAF 228
Baci    162 ADMEGEGK-T LTLSELEYIH RHKTGKMLQY SVHAGALIGG ADARQTRELD EFAAHLGLAF 220
Sulfo   153 VDMEFEDRID IKEQEYLDMI SRKTAALFSA SSSIGALIAG ANDNDVRLMS DFGTNLGIAF 212
            :*:  * :   :   *   :    **: ::   :   ** ::* :  :  : :   *: :**:**
                                                    +199

SG      231 QLVDDVLGIW GDPAATGKPA GSDLRNRKKS IPVAAAMNGT GAAAEELAAI YRGAEPLTDD 290
SA      229 QLVDDLLGIW GDPQVTGKPV YSDLRSRKKS LPIVAALTSS SPAGRELAGL YQRERPLSDA 288
Baci    221 QIRDDILDIE GAEEKIGKPV GSDQSNNKAT YPALLSLAG- ---------- ---------- 259
Sulfo   213 QIVDDILGLT ADEKELGKPV FSDIREGKKT ILVIKTLELC KEDEKKIVLK ALGNKSASKE 272
            *: **:*::  :     ***:  **  : * :       ::

SG      291 MADRAAKLIE SAGGRAWTEA EIDRHRRGAA AQLEALGLTE EQR-RPLLAL ADYVAFRNH 348
SA      289 DLVHAAELVD AVGGRAWSQT QADELLARAL HHLRSAGSVS RPI-AELGTL ARLVTHRDH 346
Baci    260 ---------- -----AK--E KLAFHIEAAQ RHLRNADVDG ----AALAYI CELVAARDH 298
Sulfo   273 ELMSSADIIK KYS-LDYAYN LAEKYYKNAI DSLNQVSSKS DIPGKALKYL AEFTIRRRK 330
                                      *    *:  :          *  : :  :  * :
                                                 +312
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Fig. 3. **Alignment of prenyltransferases.** Multiple alignment of the GGDPSase and FDPSase with the *Bacillus stearothermophilus* FDPSase and the *Sulfolobus acidocaldarius* GGDPSase are shown. Identical and similar amino acid residues are indicated by asterisks (*) and colons (:). Abbreviations: SG, *Streptomyces griseolosporeus* GGDPSase; SA, *Streptomyces argenteolus* FDPSase; Baci, *B. stearothermophilus* FDPSase; Sulfo, *S. acidocaldarius* GGDPSase. The numbers on the left and right are the residue numbers for each protein. The amino acid residues of the *B. stearothermophilus* FDPSase and *S. acidocaldarius* GGDPSase that had previously been shown to be responsible for chain length determination are underlined and numbered. The amino acid residues of the GGDPSase and FDPSase that were mutated in this study are also underlined and numbered.

They showed that the following mutations were effective for altering the product specificity, L34V, Y81H, and V157A. Among them, Y81H, which corresponds to the amino acid at the 5th position before the FARM, was reported to be the most effective (*21*). Because the amino acid at the 5th position before the FARM was the same in our two enzymes, we examined L34V and V157A. On alignment of the FDPSase of *B. stearothermophilus* with our FDPSase and GGDPSase (Fig. 3), it was revealed that L34 of the FDPSase of *B. stearothermophilus* corresponds to L50 of the GGDPSase and to S50 of the FDPSase before the FARM, and that V157 corresponds to A57 of the GGDPSase and to L58 of the FDPSase after the FARM (Fig. 3). To determine if the amino acids at these positions were responsible for the product specificity of our type I prenyltransferases, we constructed four mutated enzymes, in which the amino acids at this position were substituted for each other. The mutated enzymes, GGDPSase (L-50S), FDPSase (S-50L), GGDPSase (A+57L), and FDPSase (L+58A), were overproduced in *E. coli*, followed by purification (Fig. 1), and used for the *in vitro* assay. GGDPSase (L-50S) and FDPSase (S-50L) produced prenyl diphosphates with the same chain lengths as the wild type enzymes did (Fig. 2, lanes 5 and

Table 1. **Effect of the substrate concentration on product distribution.**

Enzyme	Substrate (μM)		Products (%)				
	DMAPP	IPP	GDP	FDP	GGDP	GFDP*	HDP**
GGDPS	125	25	3.4	4.3	91.3	1.0	ND
	25	125	2.3	1.7	95.4	0.6	ND
	25	25	3.0	1.6	94.7	0.7	ND
	5	25	3.4	2.3	92.4	1.9	ND
	25	5	2.3	3.7	92.9	1.1	ND
FDPS	125	25	8.1	88.2	3.7	ND	ND
	25	125	5.4	78.9	15.8	ND	ND
	25	25	8.1	83.1	8.7	ND	ND
	5	25	6.7	83.0	10.3	ND	ND
	25	5	8.8	86.1	5.1	ND	ND
GGDPS A+57L	125	25	6.8	89.5	3.7	ND	ND
	25	125	3.4	84.0	12.6	ND	ND
	25	25	3.9	91.2	4.9	ND	ND
	5	25	3.3	89.7	3.0	ND	ND
	25	5	7.1	90.0	7.1	ND	ND
FDPS L+58A	125	25	6.2	18.1	68.5	6.1	1.1
	25	125	4.8	3.9	66.7	23.0	1.6
	25	25	4.9	4.5	66.4	22.5	1.7
	5	25	5.0	5.5	43.7	41.4	4.4
	25	5	4.8	9.8	76.5	8.1	0.8

The enzyme was incubated with [1-^{14}C]IPP and an allylic substrate at the indicated concentrations. The reaction was terminated while more than 90% of the substrate remained. The dephosphorylated pruduct was analyzed by TLC. *Geranylfarnesyl diphosphate. **Hexaprenyl diphosphate. ND, not detected.

6). On the other hand, GGDPSase (A+57L) and FDPSase (L+58A) mainly produced FDP and GGDP, respectively (Fig. 2, lanes 7 and 8), showing that the amino acid at the 57th (58th) position after the FARM was involved in determination of the product specificity even in these type I prenyltransferases.

Previously, the amino acids at the 4th and 5th positions before the FARM were reported to play a critical role in determining the product specificity in type I prenyltransferases (*5*, *7*). To determine if these amino acids of our FDPSase and GGDPSase were also essential for determination of the product specificity, two mutated enzymes were constructed. Because the FDPSase and GGDPSase have the same amino acids at these positions (4th, Ser; and 5th, Phe), Ser at the 4th position of the GGDPSase and Phe at the 5th position of the FDPSase were replaced with Phe and Ala, respectively. The purified recombinant enzymes (Fig. 1), GGDPSase (S-4F) and FDPSase (F-5A), were used for the enzyme assay. As shown in Fig. 2, GGDPSase (S-4F) exhibited very weak catalytic activity and no spots of the product were detected under the standard conditions (Fig. 2A, lane 9). However, the product was detected on prolonged exposure, and was confirmed to be a mixture of GDP and FDP (Fig. 2A, lane 11). On the other hand, FDPSase (F-5A) mainly yielded GGDP (Fig. 2A, lane 10). These results showed that the amino acids at the 4th and 5th positions before the FARM were also responsible for the product specificities in these enzymes.

Effect of the Substrate Concentration on the Wild and Mutated Prenyltransferases—The product chain lengths of the prenyltransferases were reported to be modulated by the reaction conditions, such as the concentrations of the substrates and divalent cations (*18*, *19*). To determine if the chain lengths of the products formed by the wild type GGDPSase, wild type FDPSase, GGDPSase (A+57L), and FDPSase (L+58A) depend on the substrate concentration, the product distribution was analyzed with various substrate concentrations (Table 1). The wild type GGDPSase and FDPSase (L+58A) always produced GGDP as the main product under the employed conditions. However, FDPSase (L+58A) produced prenyl diphosphates with longer chain lengths, such as geranylfarnesyl diphosphate and hexaprenyl diphosphate, when the reaction was carried out with an excess amount of IPP. As for the wild type FDPSase and GGDPSase (A+57L), almost the same distribution pattern was observed. The main product was always FDP, and GGDP increased when an excess amount of IPP was used. These data clearly indicate that the wild type FDPSase and wild type GGDPSase are converted into a GGDPSase and a FDPSase, respectively, on amino acid substitution at the 57th (58th) position after the FARM.

Kinetic Properties of the Wild and Mutated Prenyltransferases—Although pioneering studies on the type II FDPSase of *Bacillus stearothermophilus* qualitatively demonstrated that an amino acid substitution (V157A) was effective for converting the FDPSase into a GGDPSase (*20*), the enzymatic properties of the enzyme remain unclear (*20*). Therefore, the kinetic constants of the wild type GGDPSase, the wild type FDPSase, GGDPSase (A+57L), and FDPSase (L+58A) were determined by measuring the radioactivities in hexane extracts to determine whether or not amino acid substitution at this position would affect the enzyme properties. Because the enzymatic activities of many prenyltransferases are known to be inhibited by substrates, we first determined a suitable substrate concentration for each of the

Table 2. **Kinetic properties of the wild and mutated enzymes.**

Enzyme	DMAPP			IPP		
	K_m (μM)	V_{max}*	V_{max}/K_m	K_m (μM)	V_{max}*	V_{max}/K_m
GGDPS	66.6 ± 9.7	29.7 ± 4.1	0.45	108.0 ± 24.2	18.9 ± 2.5	0.18
FDPS	31.7 ± 2.1	19.1 ± 1.2	0.60	53.1 ± 1.4	13.4 ± 0.3	0.25
GGDPS A+57L	99.2 ± 5.8	42.2 ± 2.5	0.43	97.1 ± 15.0	26.5 ± 4.3	0.30
FDPS L+58A	15.9 ± 0.6	13.2 ± 0.3	0.83	30.0 ± 4.6	5.7 ± 0.9	0.19

	FDP			IPP		
	K_m (μM)	V_{max}*	V_{max}/K_m	K_m (μM)	V_{max}*	V_{max}/K_m
GGDPS	121 ± 13.8	573 ± 66.1	4.74	35.9 ± 1.0	80.6 ± 2.0	2.25
FDPS L+58A	70.5 ± 8.0	25.2 ± 2.9	0.36	221 ± 49	26.5 ± 5.7	0.12

Kinetic constants of the wild and mutated enzymes were determined as described under "EXPERIMENTAL PROCEDURES". V_{max}* was determined as the amount of radioactivity incorporated into hexane (nmol/min/mg protein) when [1-^{14}C]IPP and the indicated allylic substrates were used. Each value is the mean ± SD of three experiments.
*U, nmol production/min.

enzymes. The enzyme activities of all the enzymes were inhibited with IPP concentrations of more than 100 μM. As for allylic substrates, the upper concentration for avoiding substrate inhibition was approximately 100 μM for all of the enzymes. Under the optimized conditions, the kinetic constants, V_{max} and K_m, for DMAPP and IPP were determined. In all cases, the substrate concentration was confirmed to be high enough for kinetic constant calculation. As shown in Table 2, the calculated V_{max}/K_m values of the enzymes that formed GGDP as the main product, the GGDPSase and FDPSase (L+58A), were almost the same for both the substrates, although FDPSase (L+58A) showed lower K_m and V_{max} values than those of the wild type enzyme. GGDPSase (A+57L), which produced FDP as the main product, also showed almost the same V_{max}/K_m values as those of the wild type FDPSase for both DMAPP and IPP, although the enzyme exhibited slightly higher K_m and V_{max} values than those of the wild type enzyme. These results suggested that the amino acid substitution introduced into the GGDPSase (A+57L) or FDPSase (L+58A) altered the product specificity without affecting either the product distribution or the enzymatic properties when DMAPP was used as the allylic substrate. However, the *V*max value of FDPSase (L+58A) was drastically reduced when FDP was used as the allylic substrate (Table 2). The calculated V_{max}/K_m values of FDPSase (L+58A) for IPP and FDP were also 19-fold and 13-fold lower than those of the wild type enzyme. These results suggested that the wild type GGDPSase is more efficient in catalyzing the elongation from FDP compared to DMAPP, while for FDPSase (L+58A) the efficiencies are similar.

DISCUSSION

In the course of a study to determine if a tetraterpene cyclase gene participating in KS-505a biosynthesis might be clustered with an octaprenyl diphosphate synthase gene, we have cloned the FDPSase gene from a KS-505a producer. Because the amino acid residues at the 4th and 5th positions before the FARM, which had previously been shown to play a critical role as to determination of the product specificity, were the same as those of the previously identified GGDPSase, we constructed several mutated enzymes based on amino acid alignment of the type II FDPSase of *Bacillus stearothermophilus*, FDPSase, and GGDPSase. Among them, the product specificities of GGDPSase (A+57L) and FDPSase (L+58A), in which the amino acid residues at the +57th (58th) position after the FARM were substituted for each other, could be interconverted without enzyme properties being affected significantly. Previously, the amino acid residue at this position was revealed to be important as to determination of the product specificity of the type II FDPSase of *B. stearothermophilus*. However, the detailed enzymatic properties of the mutated FDPSase of *B. stearothermophilus* have not been reported. Moreover, to the best of our knowledge, this is the first report on the interconversion of the product specificities of a type I FDPSase and GGDPSase through one amino acid substitution, as revealed by kinetic data. However, the amino acids at the 4th and 5th positions before the FARM of these two enzymes were also shown to play an important role in determining the product specificity. Taken together, the amino acids both before and after the FARM might harmoniously regulate the product specificity of these enzymes.

After Tarshis *et al*. (*5*) and Ohnuma *et al*. (*7*) revealed that the product specificity of type I prenyltransferases was mainly determined by the amino acid residues at the 4th and 5th positions before the FARM, studies along these lines mainly focused on the amino acids before the FARM, and not so much attention was paid to other amino acid residues. However, Ohnuma *et al.* previously reported that a type I GGDPSase of *Sulfolobus acidocaldarius* could be converted into a geranylfarnesyl (C_{25}) diphosphate or a hexaprenyl (C_{30}) diphosphate synthase by means of one to four amino acid substitutions (Fig. 3), which were randomly introduced by means of $NaNO_2$ (*6*). They showed that several amino acid residues in addition to those before the FARM were also responsible for determination of the product chain length. Moreover, Hirooka *et al.* recently reported that the product specificity of the type I GGDPSase of *S. acidocaldarius* was changed by one amino acid substitution, His114 (*22*), which corresponds to the amino acid residue at L28 after the FARM

of both the FDPSase and GGDPSase (Fig. 3). The only *E*-type prenyltransferase whose x-ray structure has been determined is an avian FDPSase (*23*). The amino acid residue located at the +57 (or +58) position after the FARM corresponds to Y179 in the avian FDPSase. The three-dimensional structure of the avian FDPSase showed that Y179 lies in a central region of the α-helix F (*23*), and is located near F112 and F113 (corresponding to the amino acid residues at the 4th and 5th positions before the FARM of both the FDPSase and GGDPSase) on α-helix D, which are known to be responsible for chain length determination. α-Helices D and F have been shown to constitute a large deep cleft, in which the chain elongation reaction takes place (*23*). Assuming that the structures of the FDPSase and GGDPSase analyzed in this study have similar tertiary structures to that of the avian FDPSase, it is reasonable that the amino acid residue located at the +57 (or +58) position after the FARM would regulate the chain termination through its bulky functional group. To determine the function of the amino acid residue at this position, however, it is indispensable to determine the crystal structures of these enzymes.

The FDPSase gene cloned from the KS-505a producer can be classified as a type I prenyltransferase, as judged from the DDXXD motif, in spite of the fact that the producer, *S. argenteolus*, is a prokaryote. *S. coelicolor* A3(*2*), the entire genome sequencing of which has been completed, has at least five putative prenyltransferase genes (*24*). All of the products also have a DDXXD motif, suggesting that actinomycetes belonging to the eubacteria usually possess only type I prenyltransferases. In this study, we could clone a prenyltransferase gene. Assuming that the KS-505a producer also has several prenyltransferase genes, similar to those of *S. coelicolor* A3(*2*), one of these genes might exist in the flanking region of a tetraterpene cyclase gene essential for the biosynthesis of KS-505a. Studies along these lines are currently in progress and the results will be reported in the near future.

This work was supported in part by a Grant-in-Aid for Scientific Research from the Japan Society for the Promotion of Science. The nucleotide sequence reported in this paper is available in the DDBJ/EMBL/GenBank databases under accession number AB083108.

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