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Protein Design of Geranyl Diphosphate Synthase. Strutural Features That Define the Product Specificities of Prenyltransferases

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Geranyl diphosphate synthase catalyzes the condensation of isopentenyl diphosphate with dimethylallyl diphosphate to give a C_{10} compound, geranyl diphosphate, which is a precursor of all monoterpenoids. However, the gene has not been isolated from any organisms. To examine the possibility that geranyl diphosphate synthase has evolved from a common ancestor of the prenyltransferase family and to predict the active site structure, we tried to convert *Bacillus stearothermophilus* farnesyl diphosphate synthase to geranyl diphosphate synthase, according to our previous findings. Several mutated farnesyl diphosphate synthases that have single amino acid substitutions before the first aspartate-rich motif were constructed. A mutated enzyme that has the replacement of serine by phenylalanine at the fourth position before the motif exclusively produced geranyl diphosphate when dimethylallyl diphosphate was used as the primer, and hardly accepted geranyl diphosphate as a primer, indicating that this mutation causes the conversion to geranyl diphosphate synthase. This result supports the idea that the product specificities of all members of the *E*-prenyltransferase family are mainly defined by a few structural features: the amino acids at the fourth position and the fifth position before the first aspartate-rich motif, and the insertion of two amino acids in the motif. This suggests that natural geranyl diphosphate synthases might have an active site structure similar to that of the mutated enzyme.

Key words: farnesyl diphosphate synthase, geranyl diphosphate synthase, product specificity, site-directed mutagenesis, structure and function of enzyme.

Prenyltransferases catalyze the consecutive condensation of isopentenyl diphosphate (IPP, C_5) with allylic diphosphates (*1-3*), and are subclassified into two groups according to the stereochemistry of the newly formed double bonds, *E*-prenyltransferases and *Z*-prenyltransferases. Each member of the prenyltransferase family produces a specific final product with a different chain length (Fig. 1), and the products are utilized as precursors of a variety of natural products such as steroids, carotenoids and prenylated proteins. Geranyl diphosphate (GPP) synthase, which is a member of the *E*-prenyltransferase family, is found in plants (*4*), and catalyzes the condensation of IPP and dimethylallyl diphosphate (DMAPP, C_5) to form a C_{10} compound. The product acts as a precursor of approximately a thousand plant fragrances (*5*). However, the corresponding gene has not been identified yet in any organism.

Recently, our group found that the product specificities of farnesyl diphosphate (FPP) synthase and geranylgeranyl diphosphate (GGPP) synthase are primarily determined by the structure around the first aspartate-rich motif [FARM, DDXX(XX)D, where D is aspartate and X is any amino acid]. This motif is highly conserved in all known *E*-prenyltransferases, and has been designated as the chain length determination (CLD) region (*6-10*). The crystal structure of avian FPP synthase supports these findings (*11, 12*). In particular, it is noteworthy that, in the case of *Sulfolobus acidocaldarius* GGPP synthase, either the replacement of a non-aromatic amino acid by an aromatic amino acid at the fourth position before the FARM or the insertion of two amino acids into the FARM causes the conversion to FPP synthase (*9*). This suggests that if these two types of mutations independently shorten the product 1 unit length, a mutated enzyme with both types of mutations should result in a product shorter than FPP, that is GPP. Thus, to test our hypothesis and to predict the active site structure of GPP synthase, we constructed such a mutated FPP synthase.

EXPERIMENTAL PROCEDURES

Materials—Precoated reversed phase thin-layer chromatography plates, LKC-18, were purchased from Whatman Chemical Separation. Precoated normal phase thin layer chromatography plates, Kieselgel 60, were purchased

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Abbreviations: IPP, isopentenyl diphosphate; GPP, geranyl diphosphate; DMAPP, dimethylallyl diphosphate; FPP, farnesyl diphosphate; GGPP, geranylgeranyl diphosphate; FARM, first aspartate rich motif; CLD, chain length determination.

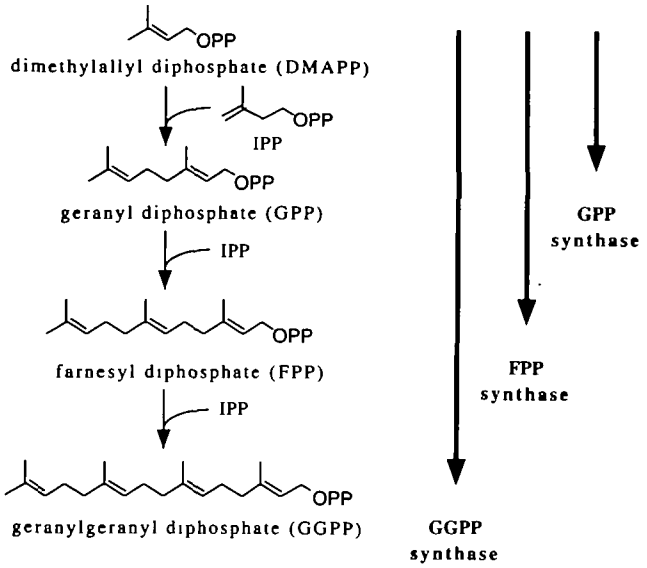

Fig. 1. **Consecutive condensation of isopentenyl diphosphates by prenyltransferases.** GPP synthase, FPP synthase, and GGPP synthase catalyze the same type of condensation, but the final products are different.

from E. Merck. (all-*E*)-FPP, GPP, and DMAPP were the same preparations as those described previously (*13*). [1-^{14}C]IPP was purchased from Amersham. pTV118N was purchased from Takara Shuzo. All other chemicals were of analytical grade. pFPS, which contains the *B. stearothermophilus* FPP synthase gene, was reported previously (*6*).

Construction of a Mutated FPP Synthase by Site-Directed Mutagenesis—Site-directed mutagenesis was performed according to Kunkel's methods (*14*). Single strand DNA coding the wild type FPP synthase gene was generated by M13KO7 helper phage infection of CJ236 cells that contained pFPS. The resulting anti-sense single strand DNA template was isolated and purified by standard methods. The synthetic sense oligonucleotides designed to produce the desired point mutations were as follows: S82F, 5′-CATACGTACTTCTTGATTCATGATGATTTG-3′; S82Y, 5′-CATACGTACTACTTGATTCATGATGATTTG-3′; S82W, 5′-CATACGTACTGGTTGATTCATGATGATTTG-3′; L83F, 5′-CGTACTCTTTCATTCATGATGATTTG-3′; L83Y, 5′-CGTACTCTTACATTCATGATGATTTG-3′; I84F, 5′-CGTACTCTTTGTTCCATGATGATTTG-3′; and I84Y, 5′-CGTACTCTTTGTACCATGATGATTTG-3′. These substituted codons are frequently used in *E. coli*. The mutated positions are indicated in bold face. New recognition sites of *Bsp*HI were introduced in some primers and are underlined. After mutagenesis, all mutations were confirmed by DNA sequencing.

Preparation of Mutated FPP Synthases—Cells of *E. coli* DH5α were transformed with plasmids carrying the mutated FPP synthase genes and cultured according to the methods described previously (*15*). The mutated enzymes were prepared by the previously described methods (*6*).

Determination of Prenyltransferase Activity—After enzymatic reaction at 55°C, enzyme activity was measured by determination of the amount of [1-^{14}C]IPP incorporated into 1-butanol-extractable polyprenyl diphosphates by the previously described method (*16*).

Product Analysis—After the enzyme reaction, the 1-butanol extracted products were treated with acid phosphatase according to the method of Fujii *et al.* (*17*). To avoid evaporation of the formed geraniol, non-radiolabeled geraniol was added as a carrier. After the hydrolysis, the resulting alcohols were extracted with petroleum ether, and then analyzed by reversed-phase thin layer chromatography on LKC-18 plates developed with acetone/H_2O (9:1), and normal phase thin layer chromatography on Kieselgel 60 plates developed with benzene/ethyl acetate (9:1). Radioactivity was detected with a Bio-image analyzer BAS2000 (FUJIFILM), and authentic standard alcohols were visualized with iodine vapor.

RESULTS

Design of Mutated FPP Synthases—To test our hypothesis and to create a mutated enzyme with GPP synthase activity, mutated FPP synthases having the active site structure of a double mutated *S. acidocaldarius* GGPP synthase that has an insertion of two amino acids in the FARM and an aromatic amino acid at the fourth position before the FARM were designed. *B. stearothermophilus* FPP synthase has a structure with four amino acids between Asp-87 and Asp-92, which corresponds to that of a two amino acid insertion into the FARM of *S. acidocaldarius* GGPP synthase, but does not have an aromatic amino acid at the fourth position before the FARM (Fig. 2). If Ser-82 of *B. stearothermophilus* FPP synthase is replaced by an aromatic amino acid, the mutated FPP synthase should have an active site structure corresponding to that of the double mutated *S. acidocaldarius* GGPP synthase. If these structures around the FARM mainly define the product specificities of all prenyltransferases, and other regions do not affect the specificity much, the mutation of FPP synthase should convert the product specificity from FPP synthase to GPP synthase. Therefore, we produced three mutated FPP synthases (S82F, S82Y, and S82W), which have a replacement with a bulky amino acid (Fig. 2). Moreover, to precisely analyze around this region, four additional mutated enzymes with a mutation at the third or second position before the FARM were produced (L83F, L83Y, I84F, and I84Y).

Measurement of Prenyltransferase Activity—The activities of the mutated FPP synthases were determined by measuring the radioactivity of 1-butanol extractable materials after the reaction of 25 μM [1-^{14}C]IPP with 25 μM DMAPP, GPP, or FPP. Figure 3 shows the activities relative to that of the wild type enzyme in the reaction with GPP as the allylic substrate. The wild type enzyme can accept both DMAPP and GPP well, but cannot accept FPP. The activity when GPP was used as the allylic substrate was 67% of that with DMAPP. On the other hand, the S82F mutant hardly accepts GPP whereas DMAPP works well as a substrate. When GPP was used, the specific activity was 7.8% of that of the wild type enzyme. These results suggest that this replacement at the fourth position before the FARM causes the conversion of the product specificity from FPP synthase to GPP synthase. S82Y did not show such a dramatic decrease in the activity toward GPP. No prenyltransferase activity was observed for S82W. In the case of mutants with a replacement at position 83 or 84, the activity of I84F was 7.2% of the corresponding activity of

the wild type when DMAPP was used. However, no significant change in the substrate specificity could be observed for the other mutants.

Product Analysis—To analyze the products of the prenyltransferase reaction, the products were hydrolyzed with acid phosphatase and the resulting alcohols were subjected to reversed-phase thin layer chromatography (Fig. 4A). In this analysis, to avoid any evaporation of C_{10} compounds such as geraniol during the procedure, non-radiolabeled geraniol was added before the hydrolysis.

With the wild type FPP synthase, the formation of a C_{10} compound was very low when 25 μM DMAPP was used as the substrate. On the other hand, as expected, mutated FPP synthase S82F exclusively produced a C_{10} compound with a very small amount of FPP. So far, neryl diphosphate, a stereoisomer of geranyl diphosphate, has also been isolated from nature. Thus, the stereochemistry of the newly formed double bond was analyzed by normal-phase TLC (Fig. 4B). The results confirmed that the product was GPP. The ratio (FPP/GPP) was 1:27. These results clearly indicated that the mutation changed the product specificity of the enzyme to change from FPP synthase to GPP synthase. We also analyzed the products of other mutated enzymes, but could not observe any change in the product specificity (data not shown).

For archaeal GGPP synthase, both the substitution at the fourth position before FARM and the insertion in FARM cause the conversion of the product specificity to FPP synthase. However, the mechanisms are different. The enzyme with the former mutation absolutely produced FPP as the final product independent of the substrate concentration, but the latter type of enzyme produced FPP in a manner dependent on the substrate concentration. If the FPP synthase activity of *B. stearothermophilus* is dictated by the insertion in FARM, the product specificity should depend on the substrate concentration. Moreover, if the

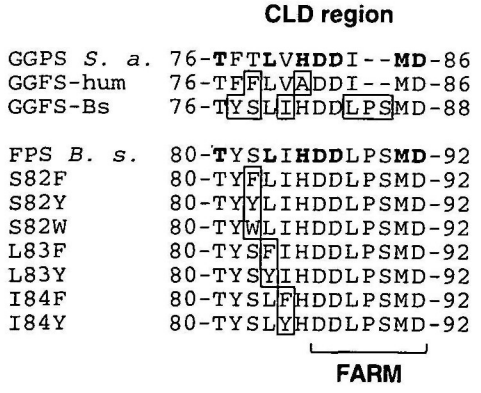

Fig. 2. **Primary structures around the CLD regions of *S. acidocaldarius* GGPP synthase and *B. stearothermophilus* FPP synthase, and the mutated enzymes.** The CLD regions of *S. acidocaldarius* GGPP synthase (GGPS *S.a.*), its mutated enzymes having FPP synthase activity (GGFS-hum and GGFS-Bs) (*9*), *B. stearothermophilus* FPP synthase (FPS *B.s.*), and its mutated enzymes constructed in this paper, are aligned. Replaced amino acids are boxed, and conserved amino acids between *B. stearothermophilus* FPP synthase and *S. acidocaldarius* GGPP synthase are indicated in bold letters.

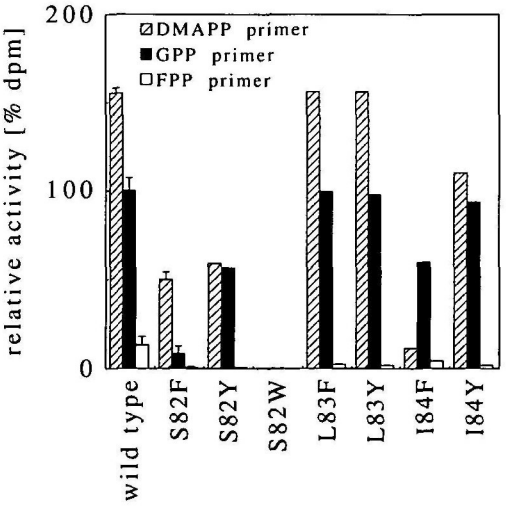

Fig. 3. **Relative prenyltransferase activity.** Enzymatic reactions were carried out using 25 μM [1-^{14}C]IPP and 25 μM of each allylic substrate. After determining the radioactivity of butanol-extracted material, the activities relative to that of the wild type enzyme in the reaction with GPP were calculated.

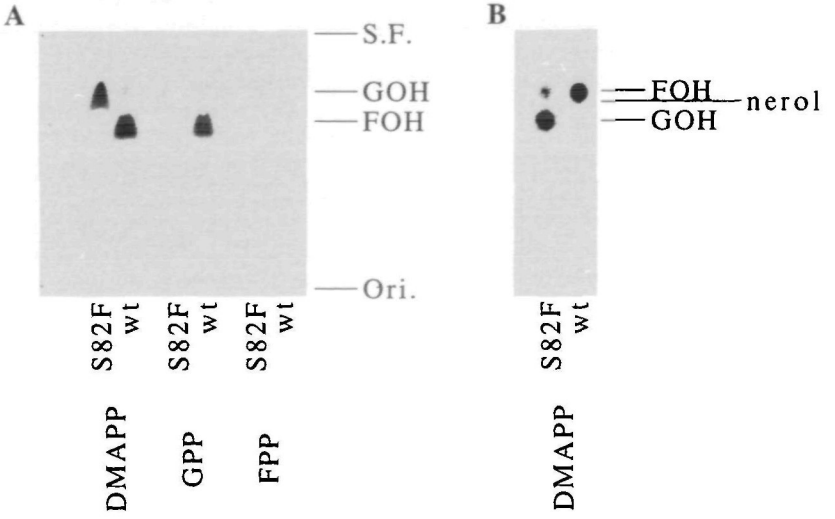

Fig. 4. **TLC autoradiochromatograms of the alcohols obtained on enzymatic hydrolysis of the products formed by mutant S82F.** A sample obtained on incubation of [1-^{14}C]IPP and each allylic substrate was analyzed by (A) reversed phase LKC-18 TLC, or (B) normal phase Kieselgel 60 TLC as described under "EXPERIMENTAL PROCEDURES." The spots of authentic standard alcohols are: GOH, geraniol; FOH, farnesol; and nerol. Ori., origin; S.F. solvent front.

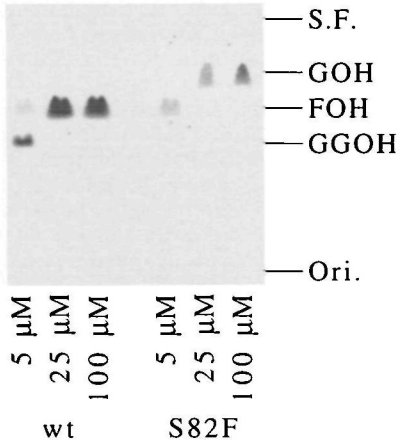

Fig. 5. **Effect of the substrate concentration on the product specificity of S82F.** The enzyme assay was performed with 25 μM [1-^{14}C]IPP and different concentrations of DMAPP, as shown. The spots of authentic standard alcohols are indicated.

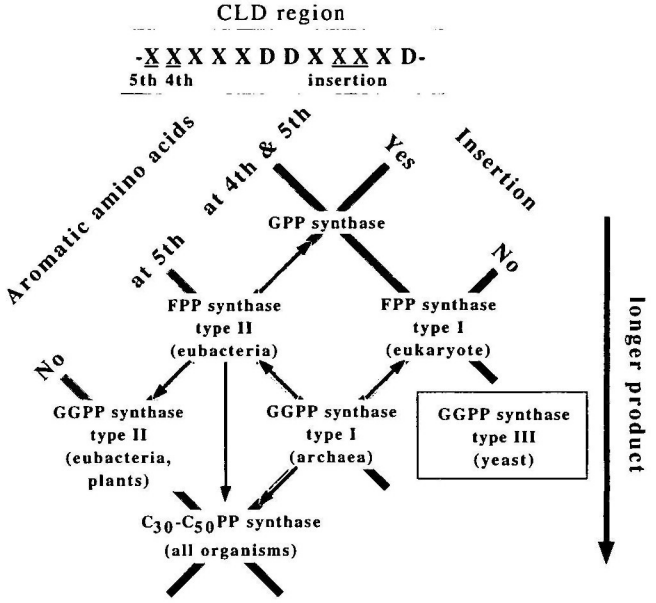

Fig. 6. **Structural requirements determining the product length.** Prenyltransferases determine the chain lengths of their final products through a combination of structures around the CLD region. Bulky amino acids at the fourth and fifth positions before the FARM, and the insertion of two amino acids into the FARM shorten the product. The effective amino acid at the fourth position must be phenylalanine. This figure enables us to determine the product specificity of all prenyltransferases from their primary structures around the CLD region. The only exception is GGPP synthase from yeast. We classified this enzyme as GGPP synthase type III. Arrows indicate the conversions that we have demonstrated so far (*6-9*).

GPP synthase activity of S82F is derived from the two structural features, the product length should also depend on the substrate concentration and the enzyme should provide a product one unit shorter than that of the wild type enzyme at any substrate concentration. Thus, the products of the wild type enzyme and S82F were analyzed with different substrate concentrations (Fig. 5). Although the wild type enzyme had the insertion, the product length did not change much with the substrate concentration. Structural differences other than in the CLD region might result in the different responsiveness to the substrate concentration. The wild type enzyme formed GGPP when 5 μM DMAPP was used, probably due to the shortage of the substrate. The S82F mutant showed a similar tendency to the wild type, and always gave a one unit shorter product than that of the wild type under the same reaction conditions.

DISCUSSION

In this paper, we have described that the *B. stearothermophilus* FPP synthase could be converted to GPP synthase by the substitution of phenylalanine by serine at the fourth position before the FARM. This result strongly supports our hypothesis that a few structural features (mainly the amino acids at the fourth and fifth positions before the FARM, and the insertion of two amino acids in the FARM) define the product specificities of short chain *E*-polyprenyldiphosphate synthases, and suggests that natural GPP synthase might have a similar active site structure.

So far, a mutated FPP synthase that produces more GPP than FPP has been reported (*18*). This mutated enzyme from *Saccharomyces cerevisiae* has a single mutation, K197E, outside the CLD region, and shows approximately tenfold lower specific activity than the wild type with either DMAPP or GPP as the allylic substrate. On the other hand, the mutated enzyme reported here, S82F, shows dramatically decreased reactivity with GPP and still accepts DMAPP well. Thus, the reason for this change in the product specificity should be different. In an avian FPP synthase, Tarshis *et al.* showed that the residue at the fourth position before the FARM directly interacts with the elongating product and regulates the chain length (*12*). It has also been suggested that the decrease in availability of allylic substrates shortens the chain length of products with some prenyltransferases (*9, 19-21*). Thus, the change in reaction product for the yeast mutant and S82F must result from the decrease in the availability of the allylic substrate and from the direct inhibition of elongation, respectively. The amino acid at the mutated site in the yeast enzyme is conserved in FPP synthases and some GGPP synthases, but there is no clear functional correlation between the product specificity and the amino acid at this position. Therefore, the amino acid at this position may not be important from the viewpoint of the product specificity of natural prenyltransferases.

Although S82F, S82Y, and S82W all have a bulky aromatic amino acid at the same position, they showed different effects: S82F acquired GPP synthase activity, whereas S82Y showed a substrate specificity similar to that of the wild type, while S82W completely lost the catalytic activity. In our previous paper, we showed that the replacement of Tyr-81 dramatically changed the product specificity, and that the size of the amino acid side chain substituted is the most important factor (*6*). In the case of these Ser-82 mutants, only the substitution to phenylalanine changed the product specificity to GPP, although the accessible surface areas of tyrosine and tryptophan are bigger than that of phenylalanine. These results indicate that at position 82 the structure of phenylalanine is just as important as its size. None of the currently known natural

FPP synthases have tyrosine or tryptophan at this position, and all type I FPP synthases have phenylalanine (Fig. 6) (*9*). Therefore, phenylalanine seems to be the only amino acid that is able to regulate the chain length at this position.

The mutants as to positions 83 and 84 did not show any changes in product chain length, although some changes in substrate specificity were observed for mutant I84F. It was reported previously that the sequence around the CLD region forms an α-helix (*11*). It is reasonable that amino acids whose side chains are positioned on the opposite surface to the substrate do not affect the determination of chain length.

So far, we have shown that the three structures in the CLD region, the fourth and fifth positions before the FARM, and the insertion of two amino acids into the FARM, are solely responsible for the regulation of the chain length. This allows us to predict the product specificities of all *E*-prenyltransferases, except for that of yeast GGPP synthases, from the primary structures (Fig. 6). For example, if a prenyltransferase has an aromatic amino acid at both the fourth and fifth positions, and has no insertions within the FARM, it should have FPP synthase activity. In fact, all eukaryotic FPP synthases (type I) have such structures (*22-27*).

The sole exception is the GGPP synthases from yeast (*28, 29*). They have neither an aromatic amino acid at the fifth position nor the insertion, but still show GGPP synthase activity. All yeast GGPP synthases have glutaminic acid in the FARM, although almost all other known prenyltransferases have an aliphatic amino acid at this position. Thus, the glutaminic acid might result in a change in the structure of the CLD region analogous to a two amino acid insertion.

To date GPP synthase has not been cloned from any plants. If the enzyme evolved from the same origin as other prenyltransferases, it may indeed have such structures around the CLD as we proposed here. One can expect GPP synthase to have a similar structure because it has been shown that polyprenyl diphosphate synthase and enzymes such as squalene cyclase that accept polyprenyl diphosphate as a substrate have similar crystal structures (*30-32*). If our hypothesis is correct, such structures must be acquired through the insertion of two amino acids into the FARM of type I FPP synthase, or through the substitution of the fourth amino acid of type II FPP synthase.

Moreover, it is known that monoterpenoids have a variety of biological functions such as repellents against insects (*33*). Our artificial FPP synthase with GPP synthase activity would be useful for the study of the biosynthesis and function of monoterpenoids.

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