A Pathway Where Polypropenyl Diphosphate Elongates in Prenyltransferase

INSIGHT INTO A COMMON MECHANISM OF CHAIN LENGTH DETERMINATION OF PRENYLTRANSFERASES

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Prenyltransferases catalyze the consecutive condensations of isopentenyl diphosphate to produce linear polypropenyl diphosphates. Each enzyme forms the final product with a specific chain length. The product specificity of an enzyme is thought to be determined by the structure around the unknown path through which the product elongates in the enzyme. To explore the path, we introduced a few mutations at the 5th, the 8th, and/or the 11th positions before the first aspartate-rich motif of geranylgeranyl-diphosphate synthase or farnesyl-diphosphate synthase. The side chains of these amino acids are situated on the same side of an α-helix. In geranylgeranyl-diphosphate synthase, a single mutated enzyme (F77G) mainly produces a C_{35} compound (Ohnuma, S.-I., Hirooka, K., Hemmi, H., Ishida, C., Ohto, C., and Nishino, T. (1996) J. Biol. Chem. 271, 18831–18837). A double mutated enzyme (L74G and F77G) mainly produces a C_{35} compound with significant amounts of C_{40} and C_{45}. A triple mutated enzyme (I71G, L74G, and F77G) mainly produces a C_{40} compound with C_{45} and C_{45}. Mutated farnesyl-diphosphate synthases also show similar patterns. These findings indicate that the elongating product passages on a surface of the side chains of the mutated amino acids, the original bulky amino acids had blocked the elongation, and the path is conserved in prenyltransferases. Moreover, the fact that some double and triple mutated enzymes can also form small amounts of products longer than C_{45} indicates that the paths in these mutated enzymes can partially access the outer surface of the enzymes.

Prenyltransferases, also referred to as polypropenyl-diphosphate synthases, are indispensable for biosyntheses of more than 20,000 naturally occurring isoprenoids and constitute a broad family of enzymes that catalyze the sequential condensations of isopentenyl diphosphate (IPP, C_{5}) with allylic prenyl diphosphates (1). These enzymes are classified into two groups according to the stereochemistry of the E or Z double bond that is formed by the condensation. Although organisms use Z-polypropenyl-diphosphate synthases only for the synthesis of dolichols for N-linked glycoprotein biosynthesis, Z-polyprenols for peptidoglycan biosynthesis in bacteria, and natural rubber, E-polypropenyl-diphosphate synthases are used for the synthesis of a vast variety of important natural isoprenoids (Fig. 1). A number of enzymes that yield (all-E)-prenyl diphosphate have been isolated from various organisms. Geranyl-diphosphate (GPP, C_{10}) synthase, found in plants (2), catalyzes the single condensation of IPP with dimethylallyl diphosphate (DMAPP, C_{5}) to give GPP. The GPP is the precursor of all monoterpenes. Farnesyl-diphosphate (FPP, C_{15}) synthase, which is one of the key enzymes of the biosynthesis of steroids, cholesterol, farnesylated proteins, sesquiterpenes, and so on, catalyzes the consecutive condensation of two molecules of IPP with DMAPP to give FPP as the ultimate product. Geranylgeranyl-diphosphate (GGPP, C_{20}) synthases are thought to be classified in two groups. One type uses DMAPP, GPP, or FPP as the allylic substrate (plant, bacteria, and archaea), and the other utilizes FPP as a substrate (fungi and animals) to give an amphiphilic molecule containing four isoprene units, GGPP, which is a precursor of diterpenes, carotenoids, geranylgeranylated proteins, and ether-linked lipids of archaea. Long E-polypropenyl-diphosphate synthases producing compounds with chain lengths from C_{30} to C_{50} are involved in respiratory quinone biosynthesis. Chicle and Gutta-percha are high molecular weight compounds like a natural rubber (3, 4). These compounds are presumed to have evolved from the common ancestor. However, what difference of amino acid causes the product specificity and how the enzymes have evolved into today’s variety of prenyltransferases are almost unknown (8, 20).

In both Sulfolobus acidocaldarius GGPP synthase and Bacillus stea rotherophilus FPP synthase, we have found that
the fifth amino acid before the first aspartate-rich motif (FARM) is extremely important for the ultimate chain length determination (21, 22). From the analysis of the amino acid, we demonstrated that the ω terminus of an elongating allylic product directly contacts with this amino acid residue, and the interaction must prevent further condensation of IPP (23). Although we examined various FPP synthases and GGPP synthases mutated at the 5th position before FARM, there was no mutated enzyme that could yield any products longer than C30 (21–23). Therefore, we hypothesized that another amino acid upstream from FARM must block the further elongation and that the alteration of this amino acid would be essential to create from GGPP synthase and FPP synthase an enzyme that produces compounds longer than C30. To demonstrate our hypothesis, we introduced mutations in the upstream region from FARM of S. acidocaldarius GGPP synthase and B. stearothermophilus FPP synthase and analyzed their products. This information should elucidate a path through which products formed by long E-polyprenyl-diphosphate synthases elongate and might elucidate a common mechanism of chain termination of long E-polyprenyl-diphosphate synthases. Furthermore, this information might enable investigators to design of a “no stop” prenyltransferase.

EXPERIMENTAL PROCEDURES

Materials—Precoated reversed-phase thin layer chromatography plates, LKC-18, and HPTLC plates RP-18 were purchased from Whatman and Merck, respectively. (All-E)-FPP, (all-E)-GGPP, GPP, and DMAPP were the same preparations as those used in the previous study (13). [1-14C]IPP was purchased from Amersham Pharmacia Biotech. pBS-GGPS and pBS-GGPSmut3 were described in our previous papers (13, 22). pEX11 was a gift by Dr. Ogura (8). All other chemicals were of analytical grade.

Construction of Mutated GGPP Synthases—Site-directed mutagenesis was carried out by the method of Kunkel (25). For the construction of pGGPS-L1, pGGPS-L2, pGGPS-L3, and pGGPS-L4, a single-strand DNA generated from pBS-GGPSmut3 (22) having the gene for the mutated S. acidocaldarius GGPP synthase (F77S) and synthetic oligonucleotides described below were used as follows: oligo-V73A, 5'-GGTGCAGCAATTGAAGCTCTTCATAC-3’; oligo-V73G, 5'-GGTGCAGCAATTGAAGGCTCTTCATAC-3’; oligo-L74A, 5'-GGTGCAGCAATTGAAGCTCTTCATAC-3’; oligo-L74G, 5'-GGTGCAGCAATTGAAGCTCTTCATAC-3’. Mutated nucleotides are indicated by bold letters. Every primer can introduce an additional restriction site of silent mutation, which is indicated by the underline. pGGPS-L5 was constructed using a single-strand DNA template from pBS-GGPS having the gene for the wild type GGPP synthase (13) and a synthetic oligonucleotide of oligo-V74G. For the construction of pGGPS-L6 and pGGPS-L7, a single-strand DNA template was isolated from Escherichia coli C326 containing pGGPS-L4, and then the second mutagenesis was carried out.
using oligo-F77A, 5'-CATACTGGTCAGTTGGCTGATGATG-3' or oligo-F77G, 5'-CATACTGGTCAGTTGGCTGATGATG-3'. These primers also contain additional restriction enzyme sites, and mutations are indicated by bold letters. Third mutations were introduced using a similar method. A single strand DNA template of pGPPS-L4, oligo-F77I-1, 5'-GGTGCAGTGGTGGAATTGTGCAATCTTGACG-3' and oligo-E72G-1, 5'-GGTGCAGCATTGGAGTTGCCGACATCTTACG-3' were used to construct pGPPS-L8 and pGPPS-L9. For the synthesis of pGPPS-L10 and pGPPS-L11, a single strand DNA template of pGPPS-L7, oligo-F77I-2, 5'-GGTGCAGTGGTGAAATTGTGCAATCTTGATCG-3' and oligo-E72G-2, 5'-GGTGCACCTGGAGTTGCCGACATCTTACG were used. Each primer also contains an additional restriction enzyme site, and mutations are indicated by bold letters. We have confirmed the mutagenesis by DNA sequencing using a model 373A DNA sequencer (Perkin-Elmer).

Preparation of Mutated GGPP Synthases and Measurement of Their Enzyme Activities—E. coli XL1-Blue was transformed with the plasmids containing mutated GGPP synthase genes and cultured according to the method described previously (26). The mutated GGPP synthases were prepared according to the previously reported method (22). In the procedures, we had used heat treatment to eliminate a majority of the mutant enzymes. Thus, we confirmed that all mutated enzymes are still thermostable (Table I). The assay mixture contained, in a final volume of 1 ml, 25 nmol of [1-14C]IPP (37 GBq/mmol), 25 nmol of the indicated allylic substrate (DMAPP, GPP, (all-E)-FPP, (all-E)-GGPP), 5 μmol of MgCl₂, 10 μmol of phosphate buffer (pH 5.8), and the same amount of indicated enzyme. This mixture was incubated at 55°C for 15 min, and the reaction was stopped by chilling quickly in an ice bath. The mixture was shaken with 3.5 ml of 1-butanol that had been saturated with H₂O. The 1-butanol layer was washed with water saturated with NaCl, and the radioactivity in the 1-butanol layer was determined with a liquid scintillation counter.

Construction of Mutated FPP Synthases—The plasmid encoding FPS-F1 was constructed by polymerase chain reaction method using pEX11 (8) as the template and four primers as follows: primer 1, 5'-ATCATCATGAACTCAAGCCGATCGTATGGGC-3'; primer 2, 5'-AAACGACCATGCGCTTTTC-3'; primer 3, 5'-CACGCAAGCTTTATATGCTG-3'; primer 4, 5'-CTTGGATCATGATGATGGT-3'. Mutated nucleotides are indicated by bold letters, and the restriction sites are indicated by an underline. An upstream fragment and a downstream fragment were amplified using the construction of the plasmid encoding FPS-F2, we used two primers as follow: primer 5, 5'-ATGGACAACGATGATTTGCG-3' and primer 6, 5'-CAACATCATGACGATCTAAAG-3'. Polymerase chain reaction was carried out using these primers and the plasmid encoding FPS-F1 as the template. The plasmid encoding FPS-F2 was formed by self-ligation of the blunt-ended amplified fragment. We have also confirmed the mutagenesis by the same way as the mutated GGPP synthase.

Preparation of Mutated FPP Synthases and Measurement of Their Enzyme Activities—E. coli XL1-Blue was transformed with the plasmids encoding mutated FPP synthases and cultured according to the method described previously (26). The mutated FPP synthases were prepared accordingly to the previously reported method (8, 21, 23). The assay mixture contained, in a final volume of 200 μl, 25 nmol of [1-14C]IPP (37 GBq/mmol), 25 nmol of the indicated allylic substrate (DMAPP, GPP, (all-E)-FPP, (all-E)-GGPP), 1 μmol of MgCl₂, 10 μmol of NH₄Cl, 10 μmol of 2-mercaptoethanol, 10 μmol of Tris-HCl buffer (pH 8.5), and a suitable amount of enzyme. After the incubation at 55°C for 20 min, the activity was determined by the 1-butanol extraction method described above.

Product Analysis—After the enzymatic reaction, the polyprenyl diposphates were extracted with 1-butanol, and then the 1-butanol was evaporated under a N₂ stream. The resulting polyprenyl diposphates were treated with acid phosphatase according to the method of Fujii et al. (27). The hydrolysates were extracted with n-pentane and analyzed by reversed-phase thin layer chromatography using LKC-18 developed with aceton/He₅O (9:1). To determine the chain length of long products, polyprenyl diposphates extracted from 10 ml of the reaction mixture were treated with the similar method and analyzed by reversed-phase thin layer chromatography using HPTLC RP-18 developed with aceton/Hexane (19:1). Authentic standard alcohols were visualized with iodine vapor, and the absolute radioactivities of the spots were measured with a Bio-image analyzer BAS2000 (Fuji). Each amount of an allylic product was determined by division of the absolute radioactivity by the number of IPPs incorporated into the product.

| Enzyme          | Specific activity (nml/min/mg) | Relative activity (DDMAPP) | Relative activity (DDGPP) | Relative activity (DDGPP) | Thermostability (%) |
|-----------------|-------------------------------|----------------------------|----------------------------|---------------------------|---------------------|
| Wild type (Cw)  | 6.4 ± 0.41                    | 100 ± 1.7                  | 100 ± 6.7                  | 100 ± 0.7                 | 95 ± 4.2                     |
| Mutant 3 (Cw, F77S) | 7.3 ± 0.35                  | 96 ± 1.0                   | 100 ± 4.8                  | 95 ± 2.7                  | 120 ± 3.4                     |
| GGPS-L1 (Cw, F73A, F77S) | 9.1 ± 0.58                 | 83 ± 7.1                   | 100 ± 6.8                  | 95 ± 3.2                  | 110 ± 11                     |
| GGPS-L2 (Cw, F73G, F77S) | 8.1 ± 0.34                 | 89 ± 8.5                   | 100 ± 4.2                  | 110 ± 7.3                | 110 ± 7.3                     |
| GGPS-L3 (Cw, F74A, F77S) | 7.1 ± 0.16                 | 90 ± 2.4                   | 100 ± 2.3                  | 110 ± 7.3                | 110 ± 7.3                     |
| GGPS-L4 (Cw, F74G, F77S) | 4.0 ± 0.13                 | 130 ± 4.0                  | 100 ± 3.3                  | 110 ± 7.3                | 110 ± 7.3                     |
| GGPS-L5 (Cw, F74S) | 9.0 ± 0.58                 | 86 ± 7.8                   | 100 ± 7.0                  | 95 ± 3.2                  | 120 ± 11                     |
| GGPS-L6 (Cw, F74G, F77A) | 6.1 ± 0.51                 | 100 ± 6.9                  | 100 ± 8.7                  | 110 ± 7.3                | 110 ± 7.3                     |
| GGPS-L7 (Cw, F74G, F77G) | 1.2 ± 0.022                | 300 ± 19                   | 100 ± 2.1                  | 110 ± 7.3                | 100 ± 4.1                     |
RESULTS

Design of Double Mutated GGPP Synthases—In our previous study, we made two mutated GGPP synthases that have the replacement of phenylalanine by serine or leucine at position 77, which is the 5th position before FARM (22). These two artificial enzymes can mainly produce geranylfarnesyl diphosphate (GFPP, C\textsubscript{25}) with a small amount of HexPP (C\textsubscript{30}). However, we failed to create an enzyme yielding a product longer than C\textsubscript{30}. We have also reported that, in \textit{B. stearothermophilus} FPP synthase, the replacement of tyrosine at the corresponding position causes a similar change in the ultimate product and that the amino acid at this position seems to contact directly with the \textit{\alpha} terminus of FPP (21, 23). Based on the fact that the mutated GGPP synthases did not yield long products, we have hypothesized that an amino acid that is situated 1 isoprene unit distant from the above mutated site directly blocks the further condensation.

Tarshis \textit{et al}. (28) have determined the three-dimensional structure of avian FPP synthase, which shows a considerable amino acid sequence similarity to \textit{S. acidocaldarius} GGPP synthase and \textit{B. stearothermophilus} FPP synthase. In the structure, the region around FARM forms an \alpha-helix. The average number of residues is 3.6 per turn in an \alpha-helix, which corresponds to 5.4 Å. This helix pitch is almost the same as the length of 1 isoprene unit of ~5.1 Å, which was calculated based on the length of carbon bonds. Thus, assuming that a growing polyprenyl diphosphate elongates along the \alpha-helix and that the elimination of the blocking side chain of the amino acid situated at one pitch of \alpha-helix distant from the fifth amino acid before FARM enables the mutated enzyme to catalyze the synthesis of products longer than C\textsubscript{30}, we constructed several double mutated GGPP synthases; the first mutation is at the fifth amino acid before FARM, the second mutation is at the third or the fourth amino acid before the first mutation (Fig. 2).

If one of the double mutated GGPP synthases can produce polyprenyl diphosphate longer than C\textsubscript{30}, it will be strong evidence for our hypothesis and will indicate that the elongating product directs to the side chain of the second mutated amino acid.

Characterization of Double Mutated GGPP Synthases—Cells of \textit{E. coli} XL1-Blue were transformed with the plasmids encoding the double mutated GGPP synthases, and the mutated enzymes were prepared according to the method reported previously. The specific activities of the mutated enzymes were determined by measuring the radioactivity in 1-butanol extractable materials of the reaction using DMAPP, GPP, FPP, or GGPP as the primer substrate (Table I). When assayed using GPP, all enzymes except for GGPS-L7 and GGPS-L4 showed comparable prenyltransferase activities. All mutated GGPP synthases and the wild type enzyme had similar relative activities for DMAPP, GPP, and FPP. On the other hand, the relative activities for GGPP based on GPP activities vary from 1.7 to 44%. The wild type enzyme hardly accepts GGPP as the primer substrate. The activity of GGPS-L5 for GGPP is also low, suggesting that the single mutation at position 74 is not enough to change the substrate specificity much. The relative activities of GGPS-L1, GGPS-L2, and GGPS-L3 are very similar to that of mutant 3 concerning total radioactive products, whereas the relative activities for GGPP of GGPS-L4, GGPS-L6, and GGPS-L7 are higher than those of GGPS-L1, GGPS-L2, and GGPS-L3. These results suggest that the mutation at position 73 has no effect on the chain length of product, whereas the leucine to glycine mutation at position 74 for mutants GGPS-L4, GGPS-L6, and GGPS-L7 is effective in changing the product specificity in such a way that L74G yields longer products than does mutant 3. To confirm these observations, the reaction products were analyzed.

Determination of the Chain Lengths of Products by the Dou-
ble Mutated GGPP Synthases—After treatment of 1-butanol extractable products with acid phosphatase, the chain length distribution of the resultant polyisoprenols and their amounts were analyzed by reversed-phase TLC. Prenyltransferases are supposed to utilize the intermediates that are initially formed and released in the reaction solution as the primer substrates again. To determine the chain length of the initially formed products as much as possible, the prenyltransferase reaction was terminated before 10% of the substrates were consumed. When the enzyme reaction was carried out using 25 μM [1-14C]IPP and 25 μM DMAPP, the wild type GGPP synthase mainly produced GGPP and did not give any products longer than GGPP (Fig. 3A). Mutant 3, which has only a mutation of F77S, mainly produced GFPP with small amounts of FPP, GGPP, and HexPP. The product distribution was similar to that previously reported (22). The product distribution did not change when the second substitutions at the position 73 were introduced as shown in the results of GGPS-L1 and GGPS-L2. On the other hand, GGPS-L3 gave a small amount of HepPP although the main product is still C25. GGPS-L4 gave C30 product as the main product in the long chain region (>C25), with a large amount of HepPP. Moreover, GGPS-L4 can form small amounts of several polypropenyl diphosphates longer than C35. These data show that the second mutation at position 74 can influence the chain length determination. Furthermore, they show that the mutated enzyme with a smaller amino acid at this position tends to produce longer products (Leu-74 < Ala-74 < Gly-74) (Fig. 3A).

We have reported in a previous paper (23) that, in the case of B. stearothermophilus FPP synthase, the chain length of product was dramatically changed by the replacement of the amino acid at the fifth amino acid before FARM and that the average chain length of products is almost inversely proportional to the accessible surface area of the substituted amino acid. Moreover, we also observed similar phenomena in S. acidocaldarius GGPP synthase. Main products of single mutated enzymes of F77S and F77G are C25 and C30, respectively (data not shown). By analogy of these results, we have constructed two more additional artificial GGPP synthases, GGPS-L6 and GGPS-L7, each of which has the replacement with a small amino acid, alanine or glycine, at position 77 in addition to L74G. When the assay was carried out using DMAPP as the primer substrate, the relative amount of HepPP formed in the reactions by GGPS-L6 is about twice greater than that by GGPS-L4. GGPS-L7 mainly forms HepPP (C30) regarding the products over C35, indicating that the bulk of the side chain of the first mutated amino acid still affects the chain length of product (Ser-77 < Ala-77 < Gly-77).
GGPS-L5, which has only one replacement at position 74 (L74G), could give a significant amount of GFPP although the relative activity for GGPP was very low (Fig. 3, Table I).

Similar product distributions were observed even when GPP, FPP, or GGPP was used as the allylic substrate (Fig. 3, B–D).

Construction of Triple Mutated GGPP Synthases and Their Product Analysis—In the previous section, we demonstrated that the double mutated enzymes can form the products longer than those formed by the single mutated enzyme. However, these enzymes could not produce polypropenyl diphasphate longer than C_{35} as the main product, although small amounts of very long products were formed as the minor products. These results indicate that some amino acid around the exit of polypropenyl diphasphate still disturbs the complete effluence of the product, suggesting that improvement around this region might confer a complete no stop enzyme that produces long polypropenyl diphosphates such as Chicle and Gutta-percha. Thus, we made four triple mutated GGPP synthases that have the third mutation at position 71 or 72 in addition to the mutations at position 74 and position 77 (Fig. 2), and we analyzed the products (Fig. 4). The third mutation is situated one pitch of \( \alpha \)-helix distant from the second mutated position 74.

When the assay was carried out using 25 \( \mu \)M DMAPP and 25 \( \mu \)M IPP, triple mutated GGPS-L10 (I71G, L74G, and F77G) increased the amount of OPP relative to HepPP, and then OPP become the major product in the products over C_{20} (OPP/HepPP = 2.3), which is 1 isoprene unit longer than that of the corresponding double mutated GGPS-L7 (OPP/HepPP = 0.34). Moreover, the other triple mutated enzyme, GGPS-L8 (I71G, L74G, F77S), increased the ratio of HepPP to HexPP (HepPP/HexPP = 2.6), compared with that of the corresponding double mutated GGPS-L4 (HepPP/HexPP = 0.29). HepPP was the major long product of GGPS-L8, and it is 1 isoprene unit longer.

Fig. 5. Analysis of very long products. After the large volume incubation with [1-\( ^{14} \)C]IPP, GPP, and the indicated double and triple mutated enzyme for 1 h, the products were analyzed by a different system using reversed-phase HPTLC RP-18. Details were described under “Experimental Procedures.” The carbon numbers of the products were indicated at left. ori., origin; s.f., solvent front.

Fig. 6. Deduced amino acid sequences of mutated bacterial FPP synthases and their product analysis. A, amino acid sequences before the first aspartate-rich motif of wild type and the mutated FPP synthases from B. stearothermophilus are shown. Y81G was reported in our previous paper (23). B, the sample from incubation of [1-\( ^{14} \)C]IPP with the indicated enzyme and the indicated allylic primer substrate was analyzed by reversed-phase LKC-18 TLC as described under “Experimental Procedures.” The carbon numbers of the products were indicated at left. ori., origin; s.f., solvent front.
L7, GGPS-L8, GGPS-L9, GGPS-L10, and GGPS-L11 were C65, detectable products of GGPS-L3, GGPS-L4, GGPS-L6, GGPS-L7, GGPS-L8, GGPS-L9, and GGPS-L10, and GGPS-L11, can produce extremely long poly-

the longest products were different. To our surprise, the longest product distribution.

The products from incubation of 25 μM 1-14CIPP with the indicated enzyme, 2.5% Triton X-100, and 2.5 μM GPP were analyzed by reversed-phase LKC-18 TLC as described under “Experimental Procedures.” The carbon numbers of the products were indicated at left. ori., origin; s.f., solvent front.

than that of GGPS-L4. On the other hand, in the triple mutated GGPS-L9 and GGPS-L11, there was no dramatic change of product distribution, although the ratio of products over C35 was slightly increased compared with the corresponding double mutated GGPS-L4 and GGPS-L7 (Fig. 4A). These results indi-

cate that the amino acid at 71 can also interact with the elongating products. Similar product distributions were ob-

erved when GPP, FPP, or GGPP was used as the primer substrate (Fig. 4, B–D).

Analysis of Products Longer Than C35—As shown in Figs. 3 and 4, the double and triple mutated GGPP synthases, GGPS-

L3, GGPS-L4, GGPS-L6, GGPS-L7, GGPS-L8, GGPS-L9, GGPS-L10, and GGPS-L11, can produce extremely long poly-

prenyl diphosphates, and the ladder of the products yielded in the reactions by some mutated enzymes appears to continue to the origin. We tried to confirm the longer limit of the product that can be synthesized by the mutated enzymes as follows. The reaction was carried out using a large volume incubation, and the products were analyzed by using a different TLC sys-

tem (Fig. 5). As a result, these eight mutated GGPP synthases were found to be able to yield the products longer than C35, but the longest products were different. To our surprise, the longest detectable products of GGPS-L3, GGPS-L4, GGPS-L6, GGPS-

L7, GGPS-L8, GGPS-L9, GGPS-L10, and GGPS-L11 were C65, C120, C110, C90, C80, C80, and C70, respectively, suggesting that the structure around the modified region affects the pro-

duction of the long products, and the paths in these mutated enzymes have partially accessed the outer surfaces of the enzymes.

Product Analysis of Mutated FPP Synthases—In the previ-

ously sections, we described that the double and triple mutated GGPP synthases can catalyze the synthesis of poly-

prenyl diphosphates much longer than those yielded by single mu-

tated enzymes. Is this effect common to all prenyltransferases? To answer this question, we constructed two kinds of double mutated bacterial FPP synthases, FPS-F1 and FPS-F2 (Fig. 6A). FPS-F1 has two replacements at positions 78 and 81, I78G and Y81G, and this corresponds to GGPS-L7. FPS-F2 has a deletion of two amino acids in FARM in addition to the two mutations of I78G and Y81G. This mutation was designed to compare eukaryotic FPP synthases and bacterial FPP syn-

thases, because the bacterial enzymes have an extra insertion of two amino acids in this region, which affects the product specificity (29). These mutated enzymes produced the poly-

prenyl diphosphates much longer than those of the wild type and a single-mutated FPP synthase, Y81G (Fig. 6B). FPS-F1 mainly gave significant amounts of products ranging from C40 to C65 in addition to three major products, GFPP, HexPP, and HepPP. When DMAPP was used as the primer, the relative amounts of the products were as follows: GPP (2.4%), FPP (10%), GGPP (3.7%), GFPP (15%), HepPP (44%), HexPP (16%), OPP (4.3%), C45PP (1.9%), C50PP (0.68%), and longer products (1.7%). The product distributions of FPS-F1 and FPS-F2 are almost similar to that of GGPS-L6, indicating that bacterial FPP synthase and archael GGPP synthase have similar structures before FARM and that the chain lengths of the products of mutated enzymes are regulated by a common mechanism. The similarity of the product distributions of FPS-F1 and FPS-F2 indicates that the extra insertion of two amino acids in FARM does not cause a significant change in the structure of the upstream region. Moreover, the ratios of longer products of the mutated FPP synthases were slightly increased as the enzyme concentration was increased. Similar observations were made in the case of mutated GGPP synthases. Ultimately, these mutated FPP synthases can catalyze the formation of up to C65 compound although the main product is still C30.

Further Improvement of GGPP Synthase—We have suc-

ceeded in converting short chain prenyl-diphosphate synthases such as GGPP synthase and FPP synthase to long chain prenyl diphosphate synthases. However, almost all of these enzymes also formed significant amounts of short products. Many prenyltransferases that catalyze formation of long polyprenyl diphosphates, including undecaprenyl diphosphate, solanesyl diphosphate, and natural rubber, require additional factors such as detergents, carrier proteins, and latex molecules (30–35). These cofactors seem to work to remove the long chain product from the enzyme effectively. Therefore, we investigat-

gated the effect of detergent on the ratio of long products. The activities of wild type and all mutated GGPP synthases were enhanced several times by the addition of 2.5% of Triton X-100, indicating that the products of these enzymes were removed efficiently. However, no change of the product distribution was observed (data not shown). Other detergents, such as Tween 80 and CHAPS, did not have any effect on the chain length of products either.

Moreover, our previous study showed that, in some prenyl-

transferases, the product distribution depends on the concen-

tration of the substrates. At a low concentration of IPP or Mg2+ ions, solanesyl-diphosphate synthase mainly produces short chain products of FPP and GGPP with significant amounts of C35 and C40, but the amounts of intermediate products are low (24). This product distribution is similar to those of our mu-

tated enzymes. When an allylic substrate is decreased or IPP is increased, the prenyltransferases increase the ratio of long products (24, 29). Thus, we examined whether these mutated GGPP synthases produced larger amounts of long products under the lower concentration of an allylic substrate with the addition of detergent. When the reaction was carried out with 2.5 μM GPP and 25 μM IPP in the presence of 2.5% Triton X-100, the main product of every mutated GGPP synthase was shifted to longer chain region (C35 to Cm) although the chain length of final product was not extended. The main products of these mutated GGPP synthases were FPP except for GGPS-L9, which produced GFPP as the main product, in the reaction with 25 μM IPP and 25 μM GPP. By contrast, the main products of GGPS-L3, GGPS-L4, GGPS-L6, GGPS-L7, GGPS-L8, GGPS-
L9, GGPS-L10, and GGPS-L11 were GFPP (73%), HexPP (41%), HexPP (65%), HepPP (29%), HepPP (28%), HexPP (59%), OPP (31%), and HexPP (34%), respectively, in reaction with 25 μM IPP, 2.5 μM GPP, and 2.5% Triton X-100 (Fig. 7). These results indicate that product distribution is affected by the substrate concentration, but cofactors or substrate concentration is not important for determination of the ultimate chain length of the products. These characters of the mutated GGPP synthases are very similar to those of natural long chain E-prenyl-diphosphate synthases such as solanesyl-diphosphate synthase (24).

**DISCUSSION**

**Chain Elongation Mechanism of Prenyltransferases**—On the basis of our present results and the three-dimensional structure of avian FPP synthase (28), we postulate a common mechanism of chain length determination in E-prenyltransferase reactions (Fig. 8). In all wild type FPP synthases or GGPP synthases, the bulky amino acid residue at the 5th position before FARM directly blocks the elongation beyond FPP or GGPP (Fig. 8A). The difference between the product specificities of FPP synthase and GGPP synthase is mainly given by the difference of the fourth amino acid before FARM and the insertion of two amino acids in the motif (29).

In the case of the single mutated GGPP synthases and FPP synthases described in Fig. 8B, the substituted amino acid at the fifth amino acid before FARM allows the mutated enzymes to catalyze the consecutive condensations of IPP beyond the native chain length of the product. An amino acid at this position must be biologically significant in the chain length determination of all natural E-prenyltransferases because all reported E-prenyltransferases that catalyze the condensation beyond GGPP have alanine at this position without exception (15–19).

The double mutated GGPP synthases, GGPS-L3, GGPS-L4, and GGPS-L6, can produce C_{30} as the main long product, which is 1 unit longer than that of the corresponding single mutated enzyme, demonstrating that, in single mutated enzymes, the side chain of Leu-74 interferes with further elongation of their products. GGPS-L7 produced C_{35} compound as the main long product, indicating that the bulk of the first mutated amino acid at the 5th position is crucial for these reactions. In general, the single mutated enzymes catalyze the condensation beyond GFPP (Fig. 8B), whereas the double and triple mutated enzymes can catalyze the condensation beyond C_{30} (Fig. 8C, 8D).
acid at position 77 still affects the product length of the double mutated enzyme. Moreover, these enzymes also can catalyze further condensations of IPP with GFPP to produce longer products (C_{50} to C_{110}). In the case of avian FPP synthase, the amino acid residues from position 94 to position 119 form an α-helix, and the region from the bottom of the α-helix to the first aspartate of FARM contains about 7 pitches of α-helix. This distance corresponds to the chain length of the hydrocarbon moiety of solanesyl diphosphate (C_{45}). Thus, these data indicate that the very long products have already protruded from the enzymes (Fig. 8C).

The triple mutated GGPP synthases of GGPS-L8 and GGPS-L10 mainly produced C_{35} and C_{40} compounds regarding the long chain products, respectively, indicating that the isoleucine situated in the pathway and directly interact with the elongating product, and we demonstrated that the replacement of small amino acids at the 5th and the 4th positions before allylic substrate binding region of GGPP synthases from S. aci-
docaldarius cause a change of allylic substrate specificity without change of the product specificity (42).

Finally, Tarshis et al. (43) has recently found that a double mutated avian FPP synthase that has two replacements with small amino acids at the 5th and the 4th positions before FARM yields mainly polypropenyl diphosphates ranging from C_{30} to C_{45} with small amounts of products longer than C_{45}. Based on the crystal structure, they have demonstrated that the growing allylic product directly interacts with the mutated amino acids. Although the second mutated position is different from those of our mutated enzymes, these results strongly support our mechanism and indicate that the structure of eukaryotic FPP synthase around the upstream region is slightly different from those of the bacterial FPP synthase and archael GGPP synthase.

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