Conversion of Product Specificity of Archaeabacterial Geranylgeranyl-diphosphate Synthase

IDENTIFICATION OF ESSENTIAL AMINO ACID RESIDUES FOR CHAIN LENGTH DETERMINATION OF PRENYLTRANSFERASE REACTION*

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Prenyltransferases catalyze the consecutive condensation of isopentenyl diphosphate with allylic diphosphates to produce prenyl diphosphates whose chain lengths are absolutely determined by each enzyme. To investigate the mechanism of the consecutive reaction and the determination of the ultimate chain length, a random mutational approach was planned. A geranylgeranyl-diphosphate synthase gene from Sulfolobus acidocaldarius was randomly mutagenized by NaNO₂ treatment to construct a library of mutated geranylgeranyl-diphosphate synthase genes on a yeast expression vector. The library was screened for suppression of a phenotype of yeast C296-LH₃, which is deficient in hexaprenyl-diphosphate synthase. Five mutants that could grow on a YEPG plate, which contained only glycerol as an energy source instead of glucose, were selected from ~1,400 mutants. All selected mutated enzymes catalyzed the formation of polyisoprenyl diphosphates with prenyl chains longer than geranylgeranyl diphosphate. Especially mutants 1, 3, and 5 showed the strongest elongation activity to produce large amounts of geranylferane-syl diphosphate with a concomitant amount of hexaprenyl diphosphate. Sequence analysis revealed that each mutant contained a few amino acid substitutions and that the mutation of Phe-77, which is located on the fifth amino acid upstream from the first asparagine-rich consensus motif, is the most effective for elongating the ultimate product. Amino acid alignment of known prenyltransferases around this position and our previous observations on farnesyl-diphosphate synthase (Ohnuma, S.-i., Nakazawa, T., Hemmi, H., Hallberg, A.-M., Koyama, T., Ogura, K., and Nishino, T. (1996) J. Biol. Chem. 271, 10087-10095) clearly indicate that the amino acid at the position of all prenyltransferases must regulate the chain elongation.

Prenyltransferases, which are essential enzymes in isoprenoid biosynthesis, catalyze the consecutive condensation of isopentenyl diphosphate (IPP) with allylic diphosphates to synthesize linear prenyl diphosphates with various chain lengths. These enzymes are classified according to the products with the longest chain length and the geometry of the double bonds that are formed by the condensations. So far, a number of prenyltransferases have been found in various organisms and characterized (1). For example, farnesyl-diphosphate synthase, which is a key enzyme in the biosynthesis of steroids, prenyquinones, farnesylated proteins, and dolichols, catalyzes the condensations of IPP with dimethylallyl diphosphate (DMAPP; C₉) and with geranyl diphosphate (GPP; C₁₀) to give farnesyl diphosphate (FPP; C₁₅) as an ultimate product. Geranylgeranyl-diphosphate (GGPP; C₂₀) synthase, whose product is a precursor of carotenoids, geranylgeranylated proteins, chlorophylls, and ether-linked lipids of archaebacteria, utilizes DMAPP, GPP, and FPP as allylic substrates to give an amphipathic molecule containing four isoprene units, GGPP (Fig. 1). Hexaprenyl-diphosphate (HPP) synthase catalyzes the consecutive condensation of IPP with E-steroechemistry to produce a C₃₀ compound (Fig. 1). Although these enzymes catalyze similar condensation reactions, they do not catalyze the condensation beyond the limit of the chain length of the product determined by their own specificities. Also, it was recently reported that these enzymes evolutionarily diverged from a common ancestor (2). Why does the condensation stop at a proper step that is determined by each enzyme? How have prenyltransferases evolved?

It has been reported that the product chain length of each enzyme is modulated by in vitro reaction conditions (3-7). In addition, several lines of evidence have been accumulated for the divergence of prenyl chain length distribution in living cells. The prenyl chain length of respiratory quinone is altered by viral infection (8) and differs from tissue to tissue (9). The dolichyl chain length in rat liver also changes on carcinogenesis (10) or aging (11, 12). On the basis of in vitro examination, Ohnuma et al. (13) and Sagami and co-workers (14) have suggested that these phenomena reflect the level of IPP and metal ions in the living cells. However, based on these pieces of information, the mechanism of the termination of the consecutive condensation has not been elucidated in terms of the molecular structure and functions of prenyltransferases.

During the past few years, the deduced amino acid sequences of FPP synthase (15-19), GGPP synthase (2, 20-23), HPP synthase (24), heptaprenyl-diphosphate synthase (25), and octaprenyl-diphosphate synthase (26) have been reported. Comparison of the primary structures has revealed that these enzymes have several conserved domains, including two.

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Chain Length Determination of Prenyltransferase

Materials—The yeast strain C296-LH输了林 (α leu2 his3 pet) and the plasmid pG3/T1 were generated by Dr. A. Tzagoloff (Cornell University). The yeast strain A451 (α can1 leu2 ura3 trp1 ar07) was provided by Dr. Nakajima (Tohoku University). The plasmid YEp335ph was kindly supplied by Dr. P. A. Edwards (University of California, Los Angeles). The plasmid Y-ctE, which contained the phosphoglycerol kinase promoter, the phosphoglycerol kinase terminator, and the ctE gene from Erwinia uredovora, was kindly given by Dr. Misawa (Kirim Brewery Co., Ltd.). Precocated reversed-phase thin-layer chromatography plates (LKC-18) were purchased from Whatman. Precocated normal-phase thin-layer chromatography plates (Kieselgel 60) were purchased from Merck. All (E)-FPP, all (E)-GGPP, GPP, and DAMPP were the same preparations as those used in a previous study (22). [1-14C]IPP was purchased from Amersham Corp. Avian myeloblastosis virus reverse transcriptase was obtained from Life Science, Inc. All other chemicals were of analytical grade.

DNA Sequence Analysis—All nucleotide sequences encoding the mutated GGPP synthases were determined by the dideoxy chain termination method using a Model 373A DNA sequencer (Perkin Elmer). DNA and deduced amino acid sequences were analyzed and compared with those of the wild-type enzyme using MacMolly Tetra genetic information processing software.

Construction of Plasmids—New HindIII sites were introduced by polymerase chain reaction-mediated mutagenesis in the 5’-upstream region and 3’-downstream region of the GGPP synthase gene from S. acidocaldarius using synthetic primers (5′-AAGAGGAAGCTTTATTGC- TACTTGAC-3′ and 5′-GATAACAGCTTTTTTTTCTCC-3′). The polymerase chain reaction-amplified product was digested with HindIII, and the 1,002-base pair fragment was ligated into the HindIII site in pBluescript KS-′ to give pBS-GGPS. Plasmid Y-ctE was digested with HindIII to cut out the ctE fragment, and then the resulting fragment that contained the phosphoglycerol kinase promoter and terminator was self-ligated to give the yeast expression vector Y-PGK. Plasmid pBS-GGPS was digested with HindIII, and then the fragment of the GGPP synthase gene was ligated with HindIII-cut dephosphorylated Y-PGK. The resultant plasmids were introduced into E. coli XL1-Blue to make a library. The plasmids were isolated from the library for further experiments.

Yeast Transformation and Screening of Mutants That Suppress pet Phenotype—Yeast transformations were carried out by the spheroplast method described by Ashby and Edwards (24). The yeast strain C296-LH输了林 was transformed with the library and spread on a leu plate using top agar (3% Bacto-agar, 0.67% yeast nitrogen base, 0.05% yeast extract, 0.05% Bacto-peptone, 1.0 M sorbitol, 2% glucose, and the appropriate amino acids). Leu + transformants were isolated and tested for complementation by plating onto YEPG (1% yeast extract, 2% ethanol, 2% Bacto-peptone, and 3% glucose) D (1% yeast extract, 2% ethanol, 2% Bacto-peptone, and 3% glucose), Dd (1% yeast extract, 2% Bacto-peptone, and 2% glucose) plate. After a 3-day culture at 30°C, the transformants that grew on the YEPG plates and formed larger colonies than C296-LH输了林 on the D plate were selected. Five positive clones were obtained from ~1,400 transformants, and every clone was analyzed. Five plasmids (Y-GGPSmut1, -2, -3, -4, and -5) were isolated from the cells.

Preparation of Mutated GGPP Synthase from Yeast—Preparation of a crude extract from yeast cells was essentially carried out by the spheroplast method described by Itoh et al. (36). S. cerevisiae C296-LH输了林,
cells harboring mutated GGPP synthase genes were grown in liquid medium at 30°C for 4 days. The cells (~400 mg) were collected by centrifugation and washed once with 800 µl of buffer A (50 mM Tris-HCl, pH 7.5, 5 mM MgCl₂, 50 mM diithiothreitol, and 1 mM sorbitol). The cells were resuspended in 1.2 ml of buffer B (50 mM Tris-HCl, pH 7.5, 5 mM MgCl₂, 3 mM diithiothreitol, and 1 mM sorbitol) and were incubated with 0.8 mg of zymolase 20T (Seikagaku Corp.) for 1 h at 30°C. The resulting spheroplasts were washed three times with buffer B and then resuspended in 1 ml of buffer C (50 mM KH₂PO₄/KOH, pH 5.8, 10 mM 2-mercaptoethanol, and 1 mM EDTA). The cell suspension was sonicated 10 times for 10 s at 2-min intervals with a Branson sonifier at the maximum output in an ice bath. The lysate was incubated at 55°C for 1 h and then centrifuged at 10,000 × g for 10 min. The supernatant was used to assay for prenyltransferase activity.

Construction of Plasmids for Expression of Mutated GGPP Synthase in E. coli—Plasmids (pBS-GGPSmut1, -2, -3, -4, and -5) were digested with HindIII, and then the DNA fragments encoding mutated GGPP synthase were recovered. The fragments were ligated with HindIII-cut dephosphorylated pBluescriptII KS⁻ to construct pBS-GGPSmut1, -2, -3, -4, and -5.

Preparation of Mutated GGPP Synthase from E. coli—E. coli XL1-Blue was transformed with each of the plasmids (pBS-GGPSmut1, -2, -3, -4, and -5) and cultured according to the methods described previously (37). The cells were harvested and disrupted by sonication in 50 mM KH₂PO₄/KOH, pH 5.8, 10 mM 2-mercaptoethanol, and 1 mM EDTA. The homogenate was heated at 55°C for 60 min and then centrifuged at 100,000 × g for 10 min. The supernatant was used as a crude enzyme to assay for prenyltransferase activity. The expression level of each mutated GGPP synthase was confirmed by SDS-polyacrylamide gel electrophoresis (12.5%) with Coomassie Brilliant Blue staining.

Measurement of Prenyltransferase Activity—The assay mixture contained, in a final volume of 1 ml, 25 nmol of [1-14C]IPP (37 GBq/mol), 25 nmol of the indicated allylic substrate (DMAPP, GGPP, all-(E)-FPP, all-(E)-GGPP), 5 µmol of MgCl₂, 10 µmol of phosphate buffer, pH 5.8, and a suitable amount of enzyme. This mixture was incubated at 55°C for 60 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 NaCl, and the radioactivity in the butanol layer was determined with a liquid scintillation counter. One unit of enzyme activity is defined as the activity required to incorporate 1 nmol of [1-14C]IPP into expanded prenyl diphosphates extracted into the 1-butanol layer.

Product Analysis—After the enzymatic reaction at 55°C with [1-14C]IPP and all-(E)-ggpp, the polypropenyl diphosphates were treated with acid phosphatase according to the method of Fujii et al. (38). The hydrolysates were extracted with pentane and analyzed by reversed-phase thin-layer chromatography using LKC-18 developed with acetone/H₂O (9:1) and by normal-phase thin-layer chromatography using Kieselgel 60 developed with benzene/ethyl acetate (9:1). Authentic standard alcohols were visualized with iodine vapor, and the distribution of radioactivity in the products was detected with a Fuji BioImage BAS2000 analyzer.

RESULTS

Effect of Wild-type GGPP Synthase on C296-LH₃—If combined with biological selection, random mutagenesis provides a

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**Fig. 2. Suppression of pet phenotype by mutated GGPP synthase.** Cells of S. cerevisiae A451 (A–C), C296-LH₃ (D–F), C296-LH₃/Y-GGPS (G–I), and C296-LH₃/Y-GGPSmut1 (J–L) were grown on YPD, D, and YEPG plates at 30°C for 3 days. The first, second, and third columns show colonies obtained from the YPD plate (A, D, G, J, and M), D plate (B, E, H, K, and N), and YEPG plate (C, F, I, L, and O), respectively. All panels are of the same magnitude. The bar in O represents 1 mm.

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**Fig. 3. TLC autoradiochromatograms of alcohols obtained by enzymatic hydrolysis of products formed by mutated GGPP synthase obtained from yeast.** The sample obtained by incubation of [1-14C]IPP and all-(E)-FPP (A) or all-(E)-GGPP (B) with the indicated enzyme from yeast C296-LH₃ was analyzed by reversed-phase LKC-18 TLC as described under "Experimental Procedures." A control experiment was carried out using the protein solution derived from untransformed C296-LH₃. Spots of authentic standard alcohols are indicated as follows: FOH, all-(E)-farnesol; GGOH, all-(E)-geranylgeraniol; GFOH, all-(E)-geranylferfarnesol; HOH, all-(E)-hexaeprenol. Ori., origin; S.F., solvent front.
powerful method for identifying important amino acid residues. The yeast strain C296-LH3 was used for screening mutated prenyltransferases whose products had prenyl chains longer than GGPP. C296-LH3 is deficient in HPP synthase and does not produce coenzyme Q6 with a hexaprenyl group. Consequently, C296-LH3 forms colonies smaller than those of the wild type when grown on a medium containing a small amount of glucose (D plate) (Fig. 2, B and E) and cannot grow on a medium containing only a nonfermentable substrate as a carbon source such as glycerol (YEPC plate) (Fig. 2F) because coenzyme Q6 is necessary for nonfermentable glycosylation.

Before screening, we examined the effect of expression of wild-type GGPP synthase from S. acidocaldarius on C296-LH3. The yeast cells expressing wild-type GGPP synthase, C296-LH3/Y-GGPS (Fig. 2K), formed colonies with a size between that of C296-LH3/YEpG3aSpH (Fig. 2H), which has the yeast HPP synthase gene, and that of C296-LH3 without any plasmids (Fig. 2E) on a D plate containing a slight amount of glucose as a carbon source. However, C296-LH3/Y-GGPS can hardly grow on a YEPC plate containing only glycerol as a carbon source (Fig. 2L). These data might indicate that S. acidocaldarius GGPP synthase could partially complement the deficiency of yeast HPP synthase. However, C296-LH3/Y-GGPS apparently formed smaller colonies compared with C296-LH3/YEpG3aSpH, and the growth characteristics of C296-LH3/Y-GGPS on a YEPC plate were quite different from those of the wild type (Fig. 2, L and I). Therefore, we were convinced that this screening system was useful.

Random Mutagenesis of GGPP Synthase Gene and Isolation of Mutants That Suppress pet Phenotype—A random chemical mutagenesis strategy using a single-stranded DNA was used to introduce mutations in the entire coding region of the GGPP synthase gene from S. acidocaldarius. Single-stranded DNA derived from pBS-GGPS was subjected to chemical mutagenesis with 1 M NaNO2 for 60 min according to the methods described by Myers et al. (35), and then a plasmid library that can express mutated GGPP synthase genes in yeast was constructed. C296-LH3 cells were transformed with the library plasmids, and then the mutants were isolated that could grow on a YEPC plate and formed colonies larger than those of C296-LH3 on a D plate as a result of the expression of functional coenzyme Q5. Five mutants (mutants 1–5) were isolated from 1,400 recombinants. Fig. 2 (M–O) shows the results of mutant 1. Mutant 1 can grow on a YEPC plate and forms colonies larger than those without the plasmid on a D plate. The colony size of mutant 1 on a D plate is apparently larger than that of the mutant containing Y-GGPS. The other four mutants also gave similar results.

Identification of Prenyltransferase Activity and Effect of Mutations—The yeast cells containing Y-GGPSmut1, -2, -3, and -5, which were obtained from the corresponding mutants, were cultured, and then their cell homogenates were prepared. The homogenates were heated at 55°C for 1 h in order to denature the enzymes derived from the host cells, and then the denatured proteins were precipitated. Using the supernatants, we analyzed the reaction products of the mutated GGPP synthases (Fig. 3). When GGPP was used as an allylic substrate, all mutants gave geranylgeranyl diphosphate (GGPP; C26), which is one isoprene unit longer than GGPP. On the other hand, the wild-type enzyme gave no detectable amount of products longer than GGPP. When FPP was used as an allylic substrate, the ratio of GGPP to GPP depended on the mutants (Fig. 3A). To analyze the mutated enzymes more precisely, they were overproduced in E. coli. The genes of the mutated GGPP synthases were integrated into pBluescriptII, and E. coli XL1-Blue cells were transformed with the plasmids pBS-GGPSmut1, -2, -3,
GFPP was 10% of the total amount of products. HPP could not be detected. Mutants 3 and 5 are similar in that they show strong GFPP synthase activities and weak HPP synthase activities. Also, these mutants produced a large amount of FPP when DMAPP or GPP was used as an allylic substrate. The amount of GPP was only different between mutants 3 and 5 when DMAPP was used. In mutant 4, the major product was GGPP, and the amount of GFPP was 15% of the total amount of products. When GPP was used, FPP was effectively produced. This might reflect the competition of GPP and FPP that was formed by a single condensation and already bound to the enzyme. Such substrate competition was observed in all mutants and the wild-type enzyme, although the magnitude differed in each case.

**DISCUSSION**

In this paper, we have tried to convert GGPP synthase from *S. acidocaldarius* to a polyisoprenyl-diphosphate synthase that catalyzes condensation beyond GGPP using random mutagenesis and phenotypic screening. An essential element of this strategy is an efficient identification of mutants of interest among a larger population of variants. For this purpose, we used a suppression system using the yeast mutant C296-LH3, which is deficient in HPP synthase. It is known that the polyisoprenyl-diphosphate synthase involved in coenzyme Q synthesis is found to be associated with the inner mitochondrial membrane (39). *S. acidocaldarius* GGPP synthase has neither an apparent transmembrane sequence nor what appears to be a typical sequence for transport into mitochondria, which contains positively charged and hydroxylated amino acids. Therefore, before screening, we were worried about whether or not the mutated GGPP synthase expressed in the cytosol of yeast could suppress the *pet* phenotype even if the enzyme had HPP synthase activity. However, success in our screening clearly shows that the overexpression of the mutated GGPP synthase in the cytosol complements the synthesis of functional coenzyme Q in mitochondria. However, it remains obscure whether the mutated GGPP synthase is transported into the mitochondria and then produces polyisoprenyl diphosphate in the mitochondria or whether the polyisoprenyl diphosphate synthesized in the cytosol moves into the mitochondria.

We obtained five mutants from 1,400 clones of the library and determined the deduced amino acid residues changed and the reaction products. These mutants showed different properties, as summarized below.

**TABLE I**

| Primer substrate | Relative activity | Product distribution |
|------------------|------------------|----------------------|
|                  | dpm              | GPP | FPP | GGPP | GFPP | HPP |
| Mutant 1 DMAPP   | 31,800           | 23.2| 8.77| 29.6 | 38.0 | 0.45|
| GPP              | 5,260            | ND  | ND  | 38.8 | 30.9 | 30.4|
| FPP              | 4,340            | ND  | ND  | 65.1 | 35.0 | ND  |
| GGPP             | 998              | ND  | ND  | ND   | 100  | ND  |
| Mutant 2 DMAPP   | 15,800           | 1.44| 0.66| 89.0 | 8.86 | ND  |
| GPP              | 7,050            | ND  | 20.3| 74.9 | 4.89 | ND  |
| FPP              | 6,080            | ND  | ND  | 89.5 | 10.5 | ND  |
| GGPP             | 379              | ND  | ND  | ND   | 100  | ND  |
| Mutant 3 DMAPP   | 24,900           | 3.40| 27.4| 16.6 | 51.6 | ND  |
| GPP              | 9,890            | ND  | 64.7| 30.4 | 19.7 | ND  |
| FPP              | 7,820            | ND  | ND  | ND   | 100  | ND  |
| GGPP             | 3,200            | ND  | ND  | ND   | 100  | ND  |
| Mutant 4 DMAPP   | 16,700           | 4.93| 4.07| 73.2 | 17.8 | ND  |
| GPP              | 7,460            | ND  | 38.4| 51.3 | 10.3 | ND  |
| FPP              | 5,650            | ND  | ND  | 85.9 | 14.1 | ND  |
| GGPP             | 551              | ND  | ND  | ND   | 100  | ND  |
| Mutant 5 DMAPP   | 23,600           | 27.1| 18.6| 12.8 | 40.4 | 1.12|
| GPP              | 9,070            | ND  | 59.3| 13.0 | 26.1 | 1.56|
| FPP              | 8,360            | ND  | ND  | 32.0 | 68.9 | ND  |
| GGPP             | 2,200            | ND  | ND  | ND   | 100  | ND  |
| Wild-type DMAPP  | 13,600           | 5.61| 0.43| 94.0 | ND   | ND  |
| GPP              | 6,600            | ND  | 17.2| 82.8 | ND   | ND  |
| FPP              | 4,650            | ND  | ND  | 100  | ND   | ND  |
| GGPP             | ND               | ND  | ND  | ND   | ND   | ND  |

a ND, not detected.
that in mutant 3 except that the amount of GPP from DMAPP (F77S and Y101H). The product distribution was similar to thase activity. Mutant 5 had alterations of two amino acids of two amino acids (F77L and V99M) and showed GFPP synthase activity. Mutant 4 contained alterations (F77S) and showed a strong GFPP synthase activity and product was GGPP. Mutant 3 contained one amino acid alteration (F77S) and showed a weak HPP synthase activity. Mutant 1 had alterations of four amino acids (F775, M85I, R199K, and D312N). The replacement of phenylalanine at position 77 seems to be essential for elongation of the ultimate product because mutant 3, which has a single mutation of F77S, catalyzes the condensation beyond GGPP. This enzyme produced similar amounts of GFPP and GGPP as main products when DMAPP or FPP was used as a priming substrate. A small amount of HPP was formed when DMAPP was used. Mutant 2 contained one amino acid alteration (F118L). This enzyme produced GFPP from any primers used, but the main product was GGPP. Mutant 3 contained one amino acid alteration (F77S) and showed a strong GFPP synthase activity and a weak HPP synthase activity. Mutant 4 contained alterations of two amino acids (F77L and V99M) and showed GFPP synthase activity. Mutant 5 had alterations of two amino acids (F775 and Y101H). The product distribution was similar to that in mutant 3 except that the amount of GPP from DMAPP was greater than that in mutant 3.

As described above, the in vitro product analysis of the mutated enzymes showed that the major product was GFPP and that overexpression of wild-type GGPP synthase partially suppressed overexpression of the phenotype of the mutant yeast. Although the lengths of the prenyl chain of coenzyme Q in the mutants have not been analyzed, it is conceivable that the length of the side chain of ubiquinone is determined by the specificity of polypropenyl-diphosphate synthase, not by 4-hydroxybenzoate polyprenyltransferase, and that coenzyme Q5 chain length determination is crucial for the determination of the ultimate chain length. It thus roseus has shown that the substitution of histidine for tyrosine at position 81, which corresponds to position 77 of E. coli, alters the structure of this enzyme and the electron transfer from NADH dehydrogenase and succinate dehydrogenase to the bc1 complex (40). So far, viral infection (8), treatment with detergent (41), and tissue differences (9) have been reported to bring about some change in the side chain length of coenzyme Q. Therefore, it would be quite interesting to determine the length of the side chain of coenzyme Q in these mutants and the rate of electron transfer in the HPP synthase-deficient strains with the mutated GGPP synthase genes.

The success in the mutagenic conversion of GGPP synthase to the hydroxybenzoate polyprenyltransferase, and that coenzyme Q5 synthases originating from organisms other than archaebacteria possess an aromatic amino acid at the position corresponding to position 77 of E. coli. B. stearothermophilus, which is concerned with the rate of electron transfer from NADH dehydrogenase and succinate dehydrogenase to the bc1 complex (40). So far, viral infection (8), treatment with detergent (41), and tissue differences (9) have been reported to bring about some change in the side chain length of coenzyme Q. Therefore, it would be quite interesting to determine the length of the side chain of coenzyme Q in these mutants and the rate of electron transfer in the HPP synthase-deficient strains with the mutated GGPP synthase genes.

The success in the mutagenic conversion of GGPP synthase to the hydroxybenzoate polyprenyltransferase, and that coenzyme Q5 synthases originating from organisms other than archaebacteria have a nonaromatic amino acid at the corresponding position. Our recent study with Bacillus stearothermophilus FPP synthase has shown that the substitution of histidine for tyrosine at position 81, which corresponds to position 77 of S. acidocaldarius GGPP synthase, brings about the conversion of FPP synthase to GGPP synthase (32). Both findings clearly demonstrate that the amino acid at this position is crucial for the determination of the ultimate chain length. It
seems likely that phenylalanine or tyrosine directly interferes with the final product so that the chain elongation cannot continue any more. Recently, Chen et al. (2) compared the amino acid sequences of all-(E)-prenyl-diphosphate synthases and proposed a phylogenetic tree. In their paper, they postulated that the bifunctional archaeobacterial GGPP synthase, which produces FPP as an intermediate, is an ancient enzyme and that HPP synthase was first separated from the bifunctionalGGPP synthase before segregation into three kingdoms, Archaea, Eubacteria, and Eucarya. After segregation into the three kingdoms, separation between FPP synthase and GGPP synthase occurred. Our finding clearly supports this evolutionary scenario.

Although we have compared the present mutations of GGPP synthase with the mutations of FPP synthase previously reported (32) and have pointed out the common importance of the aromatic amino acid at the position five amino acids before the first DDXXD motif, the mutated GGPP synthases do not show any decrease in the total reaction rate or any decrease in activity for DMAPP. These phenomena have been observed in the mutated FPP synthase. It is unclear whether or not the phenomena observed in the mutated FPP synthase directly relate to the determination of chain length. We have been analyzing the mutated GGPP synthases and the mutated FPP synthases in order to understand the common mechanism of chain length determination.

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