Conversion from Archaeal Geranylgeranyl Diphosphate Synthase to Farnesyl Diphosphate Synthase

TWO AMINO ACIDS BEFORE THE FIRST ASPARTATE-RICH MOTIF SOLELY DETERMINE EUKARYOTIC FARNESYL DIPHOSPHATE SYNTHASE ACTIVITY

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Farnesyldiphosphate (FPP) and geranylgeranyl diphosphate (GGPP) are precursors for a variety of important natural products, such as sterols, carotenoids, and prenyl quinones. Although FPP synthase and GGPP synthase catalyze similar consecutive condensations of isopentenyl diphosphate with allylic diphosphates and have several homologous regions in their amino acid sequences, nothing is known about how these enzymes form the specific products. To locate the region that causes the difference of final products between GGPP synthase and FPP synthase, we constructed six mutated archaeal GGPP synthases whose regions around the first aspartate-rich motif were replaced with the corresponding regions of FPP synthases from human, rat, Arabidopsis thaliana, Saccharomyces cerevisiae, Escherichia coli, Bacillus stearothermophilus, and from some other related mutated enzymes. From the analysis of these mutated enzymes, we revealed that the region around the first aspartate-rich motif is essential for the product specificity of all FPP synthases and that the mechanism of the chain termination in eukaryotic FPP synthases (type I) is different from those of prokaryotic FPP synthases (type II). In FPP synthases of type I, two amino acids situated at the fourth and the fifth positions before the motif solely determine their product chain length, while the product specificity of the type II enzymes is determined by one aromatic amino acid at the fifth position before the motif, two amino acids inserted in the motif, and other modifications. These data indicate that FPP synthases have evolved from the progenitor corresponding to the archaeal GGPP synthase in two ways.

Geranylgeranyl diphosphate (GGPP) synthase (EC 2.5.1.29) and farnesyl diphosphate (FPP) synthase (EC 2.5.1.10) belong to a family of prenyltransferases, and they catalyze consecutive condensations of isopentenyl diphosphate (IPP) with allylic primer substrates. Although these enzymes catalyze similar reactions, different numbers of the isoprene unit (C5) are incorporated into the final products of the reactions catalyzed by them. Moreover, the biological roles of the final products are also different. GGPP synthase catalyzes the condensation of three molecules of IPP with dimethylallyl diphosphate (DMAPP) to give a C20 compound which is utilized as a precursor for carotenoids, geranylgeranylated proteins, chlorophylls, and archaeal ether-linked lipids for the cytoplasmic membrane. FPP synthase produces a C15 compound which is a common precursor to steroids, cholesterol, farnesylated proteins, prenylated quinones, dolichols, and hemes. A number of genes of GGPP synthases (1–9) and FPP synthases (10–14) were cloned from various organisms, and their sequence alignment revealed that these enzymes showed considerable homologies (14, 15). Moreover, the phylogenetic tree proposed by Chen et al. (15) indicated that these enzymes have evolutionary originated from a common progenitor, and the enzymatic characters of archaeal GGPP synthase have suggested that archaeal GGPP synthase is near the origin (6, 7, 16). However, what difference in the amino acids between FPP synthase and GGPP synthase causes the difference in the product chain length was unknown.

Recently, our group have revealed, in both cases of FPP synthase (17) and GGPP synthase (18), that replacements of the aromatic amino acid situated at the fifth amino acid before the first aspartate-rich motif (DDXX...X D, FARM) to a nonaromatic amino acid causes a change of the product specificity. The recombinant enzymes can catalyze consecutive condensations beyond the destined limit of the original enzymes, and the chain lengths of their final products are dependent on the bulk of the side chain of the substituted amino acid (19). Moreover, hexaprenyl diphosphate synthase (20), heptaprenyl diphosphate synthase (21), and octaprenyl diphosphate synthase (22) have a small nonaromatic amino acid at the same corresponding position, indicating that the small nonaromatic amino acid primarily enables the enzymes to catalyze the condensation of IPP beyond GGPP (18). Although these results indicate that the chain length of products of GGPP synthase and FPP synthase are also determined by the amino acid at the fifth position before FARM, all GGPP synthases and FPP synthases possess an aromatic amino acid of tyrosine or phenylalanine at that position. This indicates that other amino acids regulate the final chain lengths of products formed by GGPP synthase and FFP synthase. Two questions arise. What is the difference between FPP synthase and GGPP synthase that determines the product specificity? Does the region around the FARM alone determine the product specificity of FPP synthase and GGPP synthase?

To study the mechanism of chain termination of GGPP synthase and FPP synthase and to consider an evolutionary
scheme from archaeal GGPP synthase to FPP synthase, we introduced mutations into a region around FARM of archaeal GGPP synthase and analyzed the chain lengths of their final products. This study reports that the region around FARM definitely regulates the chain lengths of the final products of GGPP synthase and FPP synthase.

EXPERIMENTAL PROCEDURES

Materials—Precast reversed-phase thin-layer chromatography plates, LKC-18, were purchased from Whatman Chemical Separation, Inc. Precast normal-phase thin-layer chromatography plates, Kieselgel 60, were purchased from Merck. (All-E)-FPP, GPP, and DMAPP were the same preparations as those described previously (6). [1-14C]IPP was purchased from Amersham Corp. pBS-GGPS and pBS-GGPSmut3 were as described in our previous study (18). 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 373A DNA sequencer. DNA and deduced amino acid sequences were analyzed and compared to those of the wild type enzyme using MacMolly/Tetra genetic information processing software.

Construction of Mutated GGPP Synthase—Site-directed mutations were introduced by the Kunkel (23) method, using single strand DNA generated from pBS-GGPS (18), which can express Sulfolobus acidocaldarius GGPP synthase, and using synthetic oligonucleotides: oligo-GGFS-hum, 5'-CATACTTTTTCTTGGTGTAGATGAGGATCGCATGGATCT-3'; oligo-GGFS-rat, 5'-CATACTTTTTCTTGGTGTAGATGAGTACTCATGATGAC-3'; oligo-GGFSat, 5'-CATACTTTTTCTTGGTGTAGATGAGTACTCATGATGAC-3'; oligo-GGFS-sc, 5'-CATACTTTTTCTTGGTGTAGATGAGTACTCATGATGAC-3'; oligo-GGFS-A2, 5'-CATACTTTTTCTTGGTGTAGATGAGTACTCATGATGAC-3'; oligo-GGFS-A4, 5'-CATACTTTTTCTTGGTGTAGATGAGTACTCATGATGAC-3'; oligo-S1, 5'-CATACTTTTTCTTGGTGTAGATGAGTACTCATGATGAC-3'; oligo-S2, 5'-CATACTTTTTCTTGGTGTAGATGAGTACTCATGATGAC-3'; oligo-S3, 5'-CATACTTTTTCTTGGTGTAGATGAGTACTCATGATGAC-3'; oligo-S4, 5'-CATACTTTTTCTTGGTGTAGATGAGTACTCATGATGAC-3'; oligo-S5, 5'-GTGACAGAGATGAGTACTCATGATGAC-3'; oligo-S6, 5'-GTGACAGAGATGAGTACTCATGATGAC-3'; oligo-S7, 5'-GTGACAGAGATGAGTACTCATGATGAC-3'. Mutated nucleotides are indicated by bold letters. Every primer contains an additionally created restriction site, which is indicated by an underline. After mutagenesis, plasmids of pGGFS-hum, pGGFS-rat, pGGFS-sc, pGGFS-A2, and pGGFS-A4 were isolated from the transformants. For the synthesis of pGGFS-Ec and pGGFS-Bs, a single strand DNA template was isolated from Escherichia coli C236 containing pGGFS-A2, and then the second mutagenesis was carried out using oligo-GGFS-Ec, 5'-ATTCCATGATGATCGGCCCGCAATGATGAC-3', oligo-GGFS-A2, 5'-ATTCCATGATGATCGGCCCGCAATGATGAC-3', or oligo-GGFS-Bs, 5'-ATTCCATGATGATCGGCCCGCAATGATGAC-3'. These primer also contain a restriction enzyme site, and mutations are indicated by bold letters. Resulting recombinant plasmids of pGGFS-Ec and pGGFS-Bs were isolated. All mutations were confirmed by DNA sequencing.

Preparation of Mutated GGPP Synthase and Measurement of Prenyltransferase Activity—E. coli XL1-Blue was transformed with each of the plasmids containing a mutated GGPP synthase gene and cultured according to the methods described previously (24). The mutated GGPP synthases were prepared according to the previously reported method (18). The expression level and the purity of each mutated GGPP synthases were confirmed by SDS-polyacrylamide gel electrophoresis (12.5%) with Coomassie Brilliant Blue staining. 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, GPP, (all-E)-FPP), 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, which had been saturated with H2O. The 1-butanol layer was washed with water saturated with NaCl, and radioactivity in the butanol layer was determined with a liquid scintillation counter. One unit of enzyme activity was 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—The chain lengths of the products were analyzed according the method described previously (17).

RESULTS

Construction of Mutated GGPP Synthases with Partial Sequences of FPP Synthases—In order to investigate the role of the region around FARM in the product specificity and the evolution of prenyltransferases, we replaced the sequence between position 77 and position 86 of GGPP synthases with that of FPP synthases. This study reports that the region around FARM definitely regulates the chain lengths of the final products of GGPP synthase and FPP synthase.

FIG. 1. Amino acid sequences of mutated archaeal GGPP synthases. Amino acid sequences around FARM of wild type and mutated GGPP synthases are shown. Mutated amino acid residues are indicated in bold letters. FARM are boxed. Mutant 3 was formed in our previous study (18).

FIG. 2. TLC autoradiochromatograms of the alcohols obtained by enzymatic hydrolysis of the products formed by the mutated GGPP synthase. The sample from incubation of [1-14C]IPP and DMAPP (A), GPP (B), or (all-E)-FPP (C) with the indicated enzyme was analyzed by reversed phase LKC-18 TLC as described under “Experimental Procedures.” Spots of authentic standard alcohols: GOH, geraniol, FOH, (all-E)-farnesol; GGOH, (all-E)-geranylgeraniol; GFOH, (all-E)-geranylfarnesol; HOH, (all-E)-hexaprenol. ori., origin; s.f., solvent front.
acidocaldarius, which is an extreme thermoacidophilic archaea, with the corresponding sequences of four eukaryotic FPP synthases from human, rat, Arabidopsis thaliana, and Saccharomyces cerevisiae, and two prokaryotic FPP synthases from E. coli, and Bacillus stearothermophilus to construct six mutated GGPP synthases (GGFS-hum, GGFS-rat, GGFS-At, GGFS-Sc, GGFS-Ec, and GGFS-Bs, Fig. 1).

Measurement of Prenyltransferase Activities of Mutated GGPP Synthases—E. coli XL1-Blue cells were transformed with the plasmids (pBS-GGPS (wild type), pBS-GGPS-mut3, pGGFS-hum, pGGFS-rat, pGGFS-At, pGGFS-Sc, pGGFS-Ec, and pGGFS-Bs) and cultured. The enzyme solutions were prepared from the cultures and heated at 55°C for 120 min in order to denature enzymes derived from the host cells because S. acidocaldarius GGPP synthase was reported to be extremely thermostable (6). After the heat treatment, we confirmed that all endogenous prenyltransferases completely lost their activities and that the heat treatment did not decrease the exogenous enzyme activity and did not change the product specificity (data not shown).

When the enzyme assay was carried out using GPP and [1-14C]IPP as the substrates, the incorporated amounts of radioactivities into 1-butanol-extractable materials were similar in all enzymes except for GGFS-Ec, which did not show any prenyltransferase activity. On the other hand, when assayed using FPP as the primer substrate, the enzyme activities of GGFS-hum, GGFS-rat, GGFS-At, GGFS-Sc, and GGFS-Bs were lower than that of the wild type enzyme. These data suggest that the activities for GGPP synthesis are decreased by the mutations (data not shown).

To confirm this, their products were analyzed by TLC after hydrolysis of a diphosphate moiety by the action of acid phosphatase (Fig. 2). In the case of the mutated GGPP synthases (GGFS-hum, GGFS-rat, GGFS-At, and GGFS-Sc) with partial sequences of eukaryotic FPP synthases, when enzyme reactions were carried out using 25 μM of DMAPP and 25 μM of [1-14C]IPP, every major product is dramatically changed from GGPP to FPP (Fig. 2A). Similar product distributions were observed in reactions using GPP as the primer substrate (Fig. 2B). However, these mutated GGPP synthases still have weak GGPP synthase activity (Fig. 2C). Because of the GGPP synthase activity, it is thought that the initially released product, FPP, is accepted again by the enzymes and converted to GGPP during a long period of incubation. To avoid this secondary reaction as much as possible, all experiments were carried out under such conditions that sufficient substrates still remained. Therefore, all the observed products should reflect the initially released products. Although these mutated GGPP synthases still have weak GGPP synthase activity, the enzymes almost become FPP synthases. These data clearly indicate that the region around FARM is responsible for the product specificity of eukaryotic type FPP synthases.

On the other hand, in the case of GGFS-Bs, a considerable amount of GGPP was formed, although the ratio of FPP was increased by the mutations. Several FPP synthases, the genes of which have been isolated from different organisms, are classified into two groups according to the sequence around FARM. One is the eukaryotic type, which is named type I FPP synthase, includes FPP synthases from human, rat, A. thaliana, and S. cerevisiae. Another is the prokaryotic type, type II FPP synthase.
synthase, has two additional amino acids inserted into FARM (DDXXXXD). These data suggest that the chain termination mechanism of the type I enzymes is different from that of the type II enzymes, although the region around FARM affects the product specificity of both types of FPP synthases.

Essential Amino Acids for Type I FPP Synthase Activity—In order to determine the essential amino acids in type I FPP synthases for displaying their product specificity, we constructed three mutated GGPP synthase, S1, S2, and S3 (Fig. 1) and analyzed their products (Fig. 3). S1 contains a single mutation of T78F, and S2 contains two mutations of F77Y and T78F. Thus, both enzymes have two proximal aromatic amino acids at the fourth and the fifth positions before FARM. When reactions were carried out using 25 μM of DMAPP and 25 μM of [1-14C]IPP, the main product of both enzymes is FPP, and the distribution patterns are similar to those of GGFS-hum, GGFS-rat, GGFS-At, and GGFS-Sc (Fig. 3A). Such similarities were also observed when GPP or FPP was used as the primer substrate (Fig. 3, B and C). These data indicate that replacement of His-81 has no influence on the chain length determination. Mutant S3, which has a single aromatic amino acid, phenylalanine, at position 78, could catalyze condensation to give the products longer than GGPP and lose its ability to produce FPP as the main product. Also, the wild type GGPP synthase, which

### Table I

| Enzyme   | Substrate Products | HexPPa |
|----------|--------------------|--------|
|          | GPP | IPP | FPP | GGPP | GFPP | HexPP |
| Wild type | 125 | 25  | 5.41 | 94.6 | ND   | ND    |
|          | 25  | 125 | 3.83 | 96.2 | ND   | ND    |
|          | 25  | 25  | 4.88 | 95.1 | ND   | ND    |
|          | 5   | 25  | 2.44 | 97.6 | ND   | ND    |
| Mutant 3 | 125 | 25  | 52.9 | 11.2 | 34.2 | 1.65  |
|          | 25  | 125 | 34.1 | 12.3 | 52.0 | 1.68  |
|          | 25  | 25  | 24.4 | 16.9 | 56.9 | 1.73  |
|          | 5   | 25  | 16.6 | 8.88 | 78.6 | 1.89  |
| GGFS-hum | 125 | 25  | 95.6 | 1.40 |
|          | 25  | 125 | 93.2 | 4.1  |
|          | 25  | 25  | 91.9 | 6.6  |
|          | 5   | 25  | 96.0 | 4.0  |
| GGFS-rat | 125 | 25  | 95.6 | 1.40 |
|          | 25  | 125 | 93.2 | 6.41 |
|          | 25  | 25  | 91.4 | 6.18 |
|          | 5   | 25  | 97.2 | 2.85 |
| GGFS-At  | 125 | 25  | 99.9 | 0.14 |
|          | 25  | 125 | 98.7 | 1.31 |
|          | 25  | 25  | 99.5 | 0.47 |
|          | 5   | 25  | 96.0 | 3.97 |
| GGFS-Sc  | 125 | 25  | 99.9 | 0.09 |
|          | 25  | 125 | 98.2 | 1.76 |
|          | 25  | 25  | 99.2 | 0.83 |
|          | 5   | 25  | 95.1 | 4.89 |
| GGFS-Bs  | 125 | 25  | 97.8 | 3.49 |
|          | 25  | 125 | 96.5 | 3.49 |
|          | 25  | 25  | 94.2 | 4.72 |
|          | 5   | 25  | 81.4 | 17.0 |
| S4       | 125 | 25  | 79.9 | 20.1 |
|          | 25  | 125 | 79.9 | 20.1 |
|          | 25  | 25  | 73.7 | 26.6 |
|          | 5   | 25  | 74.4 | 25.6 |
| S5       | 125 | 25  | 77.0 | ND   |
|          | 25  | 125 | 77.0 | ND   |
|          | 25  | 25  | 77.0 | ND   |
| S1       | 125 | 25  | 72.0 | ND   |
|          | 25  | 125 | 72.0 | ND   |
|          | 25  | 25  | 72.0 | ND   |
| A2       | 125 | 25  | 84.8 | ND   |
|          | 25  | 125 | 84.8 | ND   |
|          | 25  | 25  | 84.8 | ND   |
|          | 5   | 25  | 3.23 | 96.8 |
|          | 25  | 5   | 16.7 | 83.3 |
| A4       | 125 | 25  | 77.6 | ND   |
|          | 25  | 125 | 77.6 | ND   |
|          | 25  | 25  | 77.6 | ND   |
|          | 5   | 25  | 77.6 | ND   |

a Hexaprenyl diphosphate.
b ND, not detected.
has a single aromatic amino acid at position 77, does not produce FPP as the main product. These data clearly demonstrate that both proximal aromatic amino acids (Phe/Tyr and Phe) at the fifth and the fourth positions before FARM are necessary and sufficient to terminate the reaction after the step for FPP formation.

Essential Amino Acids for Type II FPP Synthase Activity—Although we demonstrate that the product specificity of type I FPP synthase is donated by the two proximal amino acids at the fourth and the fifth positions before FARM, type II FPP synthases do not have phenylalanine at the fourth position. This clearly indicates that amino acids contributing to chain termination are different between type I and type II FPP synthases. Thus, we constructed four mutated GGPP synthases (A2, A4, S4, and S5) (Fig. 1) to investigate the mechanism of type II enzyme.

A large number of FPP synthases have been cloned and sequenced with a variety of organisms. These data are summarized in Table I. Of note is the observation that the amino acid at position 4 before the FARM sequence is always threonine, while the amino acid at position 5 before FARM is an aromatic amino acid in type I FPP synthases, whereas type II FPP synthases do not have Phe at position 4.

The CLD region of all eukaryotic FPP synthases is boxed. The sequence numbers indicate residue positions in the primary sequences. Numbers in parentheses indicate the GenBank accession nos. for the sequences as follows: S. acidocaldarius FPP synthase, C83989; M. thermoautotrophicum FPP synthase, A26079; H. sapiens FPP synthase, M15410; A. thaliana FPP synthase, A64171; S. cerevisiae FPP synthase, J05091; E. coli FPP synthase, D00694; B. stearothermophilus FPP synthase, D13293; A. thaliana GGPP synthase, L25813; and E. coli octaprenyl-diphosphate synthase, U18997. The GenBank accession nos. for the sequences are as follows: S. acidocaldarius GGPP synthase, D28748; M. thermoautotrophicum GGPP synthase, S75695; M. jannaschii GGPP synthase, S75695; H. sapiens FPP synthase, J05262; R. rattus FPP synthase, M34477; A. thaliana FPP synthase, D00694; B. stearothermophilus FPP synthase, D13293; A. thaliana GGPP synthase, L25813; L. albus GGPP synthase, U15778; C. roseus GGPP synthase, X92893; N. crassa GGPP synthase, U20940; E. uredovora GGPP synthase, D90087; E. herbicola GGPP synthase, M87280; R. capsulatus GGPP synthase, X52291; S. cerevisiae FPP synthase, J05091; E. coli GGPP synthase, D00694; B. stearothermophilus FPP synthase, D13293; A. thaliana GGPP synthase, L25813; L. albus GGPP synthase, U15778; C. roseus GGPP synthase, X92893; N. crassa GGPP synthase, U20940; E. uredovora GGPP synthase, D90087; E. herbicola GGPP synthase, M87280; R. capsulatus GGPP synthase, X52291; S. cerevisiae FPP synthase, U31632; S. cerevisiae HPP synthase, J05547; B. stearothermophilus heptaprenyl-diphosphate synthase, D49976; and E. coli octaprenyl-diphosphate synthase, U18997.
almost similar to that of the wild type enzyme. These data indicate that F77Y, which is situated at the fifth position before FARM, partially contributes to acquiring FPP synthase activity, and more substitutions are also necessary to gain a complete FPP synthase activity.

Mutants S4 and S5 have an insertion of two amino acids between positions 84 and 85 and a substitution of leucine for isoleucine at position 84. When assayed using 25 μM of an allylic substrate, 25 μM of IPP, and these enzymes, the ratio of FPP in the total products is slightly increased by the mutations.

**Effect of Substrate Concentration in Mutated GGPP Synthases**—It has been reported that the product distribution of natural prenyltransferases is modulated by *in vitro* reaction conditions, such as the concentrations of substrates and metal ions (25–28), and it is suggested that these phenomena reflect the affinity between the enzymes and the substrates that bind to metal ions (26, 27). Moreover, we recently found that, when reactions were carried out using low concentrations of IPP or high concentrations of an allylic substrate, *S. acidocaldarius* GGPP synthases that have mutations in the region from position 170 to position 173 yield large amounts of short chain products than does the wild type enzyme.5 Thus, in order to investigate whether the chain length of the product yielded by the present mutated GGPP synthases depends on the substrate concentration, we analyzed the products formed under different substrate concentrations (Table I).

Wild type GGPP synthase always gives GGPP as the main product, and the distribution of products does not vary much within the range of the substrate concentration that is used. In the case of GGFS-hum, GGFS-rat, GGFS-Sc, GGFS-At, S1, and S2, the main product is FPP in any case, although the ratio of GGPP to FPP is slightly increased as the concentration of IPP increases or the concentration of GPP decreases.

On the other hand, the product distributions of GGFS-Bs, S4, and S5 are dramatically changed with the substrate concentration used. In GGFS-Bs, the ratio of FPP in the total products seems to depend on the IPP concentration. For example, the ratio observed in the reaction with 125 μM of IPP is 54.4%, whereas the ratio in the reaction with 5 μM of IPP is 100%. Thus, this mutant can almost be FPP synthase. As shown in Fig. 4, no significant difference was observed in the product distribution between the wild type enzyme and S4 or S5 when 25 μM of IPP and 25 μM of an allylic substrate were used. However, when assayed at different concentrations, the difference between these mutants and the wild type became clear. When 125 μM of GGPP and 25 μM of IPP were used, the relative amounts of FPP in the case of S4 (23.0%) and S5 (15.3%) were greater than in the case of the wild type (5.4%). Further elongation was also observed when 25 μM of GGPP and 5 μM of IPP were used. These data mean that the insertion in the FARM also independently makes the enzyme in favor of the formation of FPP in a manner dependent on the substrate concentration. Moreover, the amount of FPP formed in the reaction by A2 also depends on the substrate concentration. However, the effect is weaker than those observed in S4 and S5. On the other hand, the mutations of T78S and V81I, which are observed in A4, did not affect the product distribution in any case. These data indicate that the mutation of the fifth amino acid before FARM and the two-amino acid insertion in FARM are necessary for terminating the reaction after the step for FPP formation.

Discussion

In this work, we described that the replacement of a few amino acids around FARM and the insertion in FARM of archaeal GGPP synthase cause a change of the enzyme activity so that it is converted into FPP synthase. We also indicated that FPP synthases are classified into two types according to the sequences around FARM and that the chain termination mechanisms of these two types of FPP synthases are different. These findings suggest that FPP synthases evolved from the same origin in two ways. If archaeal GGPP synthase is near the origin, type I FPP synthase activity should have been conferred by a substitution of the amino acid at the fourth amino acid before FARM. Regarding type II FPP synthases, replacement of the amino acid at the fifth amino acid before FARM, insertion of two amino acids in FARM, and other modifications have occurred to change product specificity (Fig. 5A).

Almost all organisms have three kinds of (all-E)-polyprenyl diphosphate synthases (FPP synthase, GGPP synthase, and long chain polyprenyl diphosphate synthase). In previous studies, our group has reported that mutated FPP synthase from *B. stearothermophilus* and mutated GGPP synthase from *S. acidocaldarius* that have the substitution for a small amino acid at the fifth position before FARM have long chain polyprenyl diphosphate synthase activity (17, 18). On the basis of these results of our studies, we conclude that the region from the fifth position before FARM to the end of FARM essentially determines the final products of (all-E)-polyprenyl diphosphate synthases, and we propose to designate this region as the CLD (chain length determination) region (Fig. 5B).

Recently, Tarsisia *et al.* (29) reported the three-dimensional structure of avian FPP synthase. In the structure, the CLD region forms an α-helix, and the distance between the first aspartate (Asp-117) in FARM and the fifth or the fourth amino acid before FARM is about 10–12 Å. This distance is similar to the length of the hydrocarbon moiety of FPP. These data strongly suggest that these amino acids must directly block further elongation.

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