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# Effects of Random Mutagenesis in a Putative Substrate-Binding Domain of Geranylgeranyl Diphosphate Synthase upon Intermediate Formation and Substrate Specificity[1]

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**Archaeal geranylgeranyl diphosphate (GGPP) synthase catalyzes the consecutive condensation of isopentenyl diphosphate (IPP) with allylic diphosphates to produce GGPP with significant amounts of intermediates. To obtain information about the amino acids involved in the condensation and the release of intermediates, we randomly mutagenized two proximal regions, I and II, of the *Sulfolobus acidocaldarius* GGPP synthase gene and created two degenerate libraries, I and II, respectively. Regions I and II correspond to amino acid residues 170-173 and 166-168, respectively. The prenyltransferase activities of about 200 clones were analyzed using the *in vivo* red-white system and the conventional *in vitro* assay. Although, in library I, no mutated enzymes that failed to catalyze the formation of GGPP were found, as assayed with the red-white system, almost all the mutated enzymes exhibited weak GGPP synthesis activity, and many produced large amounts of intermediates. The formation of intermediates increased as the concentration of IPP was decreased or as the concentration of the allylic substrate was increased. These phenomena can be regarded as a reflection of the increased $K_m$ for IPP and the decreased affinity for products including intermediates. On the other hand, no mutants from library II showed such changes. These results suggest that the region from 170 to 173 is concerned in the recognition of both IPP and allylic diphosphates, and that the change in responsiveness to prenyl diphosphates causes a change in intermediate formation.**

**Key words: enzyme mechanism, farnesyl diphosphate, geranylgeranyl diphosphate synthase, isoprenoids, prenyltransferase.**

Prenyltransferases comprise a broad group of enzymes catalyzing the consecutive condensation of isopentenyl diphosphate (IPP) with allylic diphosphates to yield prenyl diphosphates with various chain lengths. These enzymes are classified according to the chain length of the final product and the geometry of the double bond that is formed on the condensation.

Geranylgeranyl diphosphate (GGPP) synthase, a member of the prenyltransferase family, is a key enzyme in the biosynthesis of carotenoids, geranylgeranylated proteins, chlorophylls, ether-linked lipids of archaea and so on. All GGPP synthases catalyze the condensation of IPP to produce GGPP ($C_{20}$). In spite of the similarity of the deduced amino acid sequences of cloned GGPP synthases, the degrees of intermediate formation and substrate specificity differ depending on the origin. For example, eubacterial GGPP synthase catalyzes the consecutive condensation of IPP with dimethylallyl diphosphate (DMAPP, $C_5$), geranyl diphosphate (GPP, $C_{10}$), and farnesyl diphosphate (FPP, $C_{15}$), to yield GGPP ($C_{20}$) as the ultimate product. The GGPP synthases from cow (*1*) and yeast (*2*) only catalyze the single condensation of IPP with FPP and hardly accept DMAPP or GPP as a priming substrate. Archaeal GGPP synthase, which has been assumed to be near the ancestor of all prenyltransferases (*3*), can accept either DMAPP, GPP, or FPP as a priming substrate, and releases a considerable amount of FPP as an intermediate (*4-6*). Thus, this archaeal type enzyme has been thought to be responsible for the formation of both FPP and GGPP in archaeal cells. However, which regions and amino acid residues of GGPP synthases are related to the formation of intermediates and the substrate recognition are unclear.

It has been reported that prenyltransferases other than GGPP synthases also produce small amounts of intermediates and that the relative amounts of the intermediates changes with the reaction conditions in some cases (*7-9*). On the basis of *in vitro* experiments involving solanesyl diphosphate synthase and dehydrodolichyl diphosphate synthase, Ohnuma *et al.* (*7*) and Matsuoka *et al.* (*8*) have reported that these phenomena reflect the levels of IPP and

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Abbreviations: DMAPP, dimethylallyl diphosphate; FPP, (all-*E*)-farnesyl diphosphate; GGPP, (all-*E*)-geranylgeranyl diphosphate; GPP, geranyl diphosphate; IPP, isopentenyl diphosphate; MBP, maltose binding protein.

metal ions. However, different prenyltransferases show different tendencies as to the production of intermediates depending on the concentrations of IPP and metal ions, and it is unclear why such differences occur and which amino acids or regions are related to the product distribution.

Moreover, it is well known that prenyltransferases that catalyze chain elongation beyond GGPP require a priming substrate with a suitable chain length such as FPP. Thus, in order to obtain information about the amino acid residues involved in the phenomena of intermediate formation and substrate recognition, and to elucidate their roles, we introduced site-directed random mutations into two regions of archaeal *Sulfolobus acidocaldarius* GGPP synthase, which have been thought to be parts of the putative binding region for the hydrocarbon moieties of the substrates.

## EXPERIMENTAL PROCEDURES

*Materials*—Precoated reversed-phase thin-layer chromatography plates, LKC-18, were purchased from Whatman Chemical Separation, Inc. Precoated normal-phase thin-layer chromatography plates, Kieselgel 60, were purchased from E. Merck. (all-*E*)-FPP, GPP, and DMAPP were the same preparations as used in the previous work (*4*). [1-$^{14}$C]IPP was purchased from Amersham. All other chemicals were of analytical grade.

*Random Mutagenesis of the GGPP Synthase Gene*—A *Hin*dIII/*Kpn*I fragment derived from plasmid RV21-2 (*4*), which contained the *S. acidocaldarius* wild-type GGPP synthase gene, was subcloned into pUC119, yielding pHK119. Two libraries, I and II, which contained random mutations of nucleotides encoding two proximal regions, I (amino acid residues 170–173) and II (amino acid residues 166–168), of the GGPP synthase, were constructed using PCR (Fig. 2). In order to introduce random mutations into region I, we prepared four primers; primer 1, 5′-AAGAGGGTTACCCACAGTCC-3′; primer 2, 5′-CATGTCTAGATATTCCTGCTCCTTTAT-3′; primer 3, 5′-AATATCTAGANNNNNNNNNNCGTAAGACAGCTGCATT-3′; and primer 4, 5′-CCTAAGATATCGTCAACAATC-3′, in which the newly introduced restriction sites are underlined. An upstream fragment and a downstream fragment were amplified using the combination of primers 1 and 2, and that of primers 3 and 4, respectively. The upstream fragment obtained from the digestion with *Eco*065I and *Xba*I, and the downstream fragment obtained on digestion with *Xba*I and *Eco*RV were gel-purified and ligated back into the *Eco*065I and *Eco*RV sites of the original pHK119 plasmid so that an unmutated portion of region I was replaced with PCR-mediated random sequences. *Escherichia coli* DH5$\alpha$ was then transformed with the reconstituted plasmids. Library II, which contained random mutations in region II, was constructed by the same procedures using two primers in addition to primers 1 and 4; primer 5, 5′-CATGTCTAGANNNNNNNNCTCCTTTATATCAATTC-3′; and primer 6, 5′-ATATCTAGACATGATCTCACGTAA-3′. A few hundred clones were isolated from the two libraries and analyzed.

*In Vivo Analysis of GGPP Synthase Activity*—Forty and 149 plasmids were isolated from libraries I and II, respectively. Competent cells containing pACYC-IB (*4*), which has two carotenogenic genes, *i.e.* those of phytoene synthase (*crtB*) and phytoene desaturase (*crtI*), and a replication origin compatible for ColE1 derived plasmids, were transformed with the plasmids and spread on LB plates containing tetracycline (50 $\mu$g/ml) and ampicillin (50 $\mu$g/ml). Because the competent cells expressed phytoene synthase (CrtB) and phytoene desaturase (CrtI), a clone transformed with a plasmid that shows GGPP synthase activity produced lycopene, thereby becoming red (Fig. 1). In the case of library I, eight white colonies and 32 red ones were obtained. In the case of library II, eight clones formed white colonies and the remaining clones gave red ones. The coding regions of all mutated GGPP synthases were sequenced by the dideoxy chain termination method.

*Preparation of Crude Mutated GGPP Synthase*—Cells of *E. coli* DH5$\alpha$ were transformed with the 15 and 32 plasmids derived from the red colonies of libraries I and II, respectively, and cultured according to the method described previously (*10*). The cells were harvested and disrupted by sonication in 50 mM $KH_2PO_4$/KOH buffer, pH 5.8, containing 10 mM 2-mercaptoethanol and 1 mM EDTA. Each homogenate was heated at 55°C for 120 min and then centrifuged at 100,000 × *g* for 10 min. The supernatant was used as the crude enzyme. The expression levels of all mutated FPP synthases were confirmed by sodium dodecyl sulfate polyacrylamide electrophoresis (12.5%) with Coomassie Brilliant Blue staining, using homogeneously purified wild type GGPP synthase as a standard (data not shown).

*Preparation and Purification of Geranylgeranyl Diphosphate Synthase Fused with Maltose Binding Protein (MBP), and Mutations of It*—The *Sulfolobus acidocaldarius* geranylgeranyl diphosphate synthase gene was amplified by

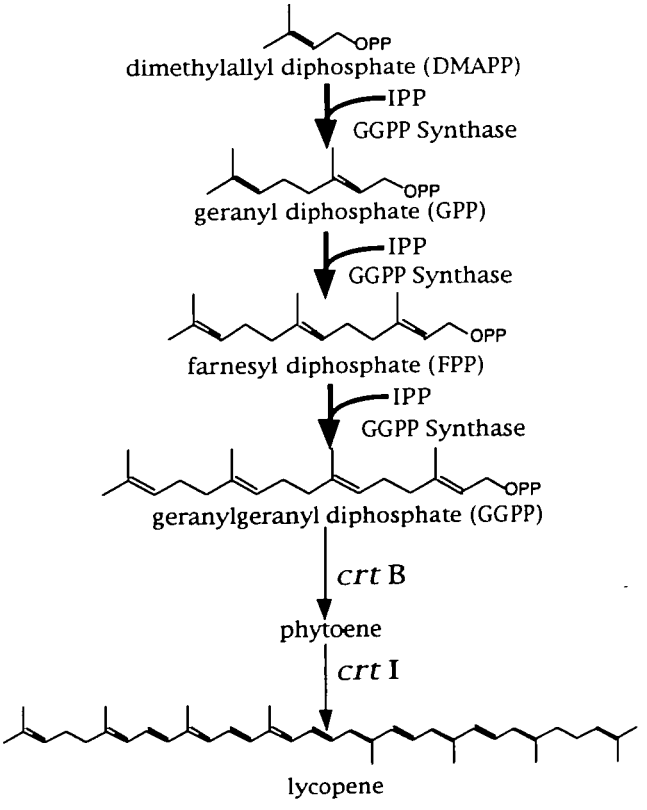


Fig. 1. **Geranylgeranyl diphosphate synthase reaction and lycopene formation.**

PCR using pGGPS1 (*4*) as a template and synthetic oligonucleotides as primers, whose nucleotide sequences were 5′-CGCGGATCCATGAGTTACTTTGACAA-3′ and 5′-GGGAATTCTTATTTTCTCCTTCTTA-3′. The amplified DNA fragment was cut with *Eco*RI. After blunt-ending, the fragment was digested with *Bam*HI. The resulting fragment was ligated with the pMal-c2 vector cut with *Bam*HI and *Hin*dIII, which was blunt-ended. *E. coli* DH5$\alpha$ was transformed with the resultant plasmid, which was named pMalcGG2. The transformants were cultured in 2×YT medium at 37°C until $OD_{600}$ reached about 0.5. After the addition of 1 mM IPTG, the cells were collected and sonicated. Purification of the supernatant was carried out on an amylose resin (Bio-Rad) affinity column. The purified MBP-fused GGPP synthase was heated at 55°C for 30 min, filtrated through a Maeshori Disk (Tosoh), and then gel-filtrated on a Nick Column (Pharmacia) to change the buffer to Factor Xa buffer containing Tween 20. The MBP-fused GGPP synthase was cut according to the previously reported method (*11*) except that all solutions additionally contained 0.25% of Tween 20, and the lysis buffer contained 0.1 mM phenylmethylsulfonyl fluoride.

For the preparation of mutated GGPP synthases, I-8, I-11, I-28, and I-40, the *Pma*C1-*Eco*T22I fragment of pMalcGG2 was exchanged with the corresponding fragments from the I-8, I-11, I-28, and I-40 plasmids, named pMalcGG2m-I-8, pMalcGG2m-I-11, pMalcGG2m-I-28, and pMalcGG2m-I-40, respectively. The MBP-fused mutated GGPP synthases were expressed and purified as described above. After purification of the wild type enzyme and all mutated enzymes, we confirmed their homogeneities by sodium dodecyl sulfate polyacrylamide electrophoresis (12.5%) with Coomassie Brilliant Blue staining (data not shown). A DC protein assay kit (Bio-Rad) was used for all protein concentration determinations.

*Measurement of Prenyltransferase Activity*—The assay mixture contained, in a final volume of 1 ml, the indicated amount of [1-$^{14}$C]IPP (2.00 GBq/mmol or 37 GBq/mol), 25 nmol of the indicated allylic substrate [DMAPP, GPP, or (all-*E*)-FPP], 5 $\mu$mol of $MgCl_2$, 10 $\mu$mol of phosphate buffer (pH 5.8), and the indicated amount of enzyme. This mixture was incubated at 55°C for 5 min, and the reaction was stopped by chilling in an ice bath. The mixture was shaken with 3.5 ml of 1-butanol which had been saturated with $H_2O$. The 1-butanol layer was washed with water saturated with NaCl, and then the radioactivity in the 1-butanol layer was determined.

*Product Analysis*—After enzymatic reaction at 55°C, the polyprenyl diphosphates were extracted with 1-butanol, and the 1-butanol was then evaporated under a $N_2$ stream. The resulting polyprenyl diphosphates were treated with acid phosphatase according to the method of Fujii *et al.* (*12*). The hydrolysates were extracted with pentane, and then analyzed by reversed phase thin layer chromatography, using LKC-18, developed with acetone/$H_2O$ (9 : 1), and normal phase thin layer chromatography, using Kieselgel 60, developed with benzene/ethyl acetate (9 : 1, data not shown). Authentic standard alcohols were visualized with iodine vapor, and the distribution of radioactivity was analyzed with a Bio-image analyzer BAS2000 (Fuji Film).

## RESULTS

*Region-Directed Random Mutagenesis of the GGPP Synthase Gene of Archaeal S. acidocaldarius*—We constracted two degenerate libraries with mutations in the two proximal regions of the GGPP synthase gene from *S. acidocaldarius* (Fig. 2). Regions I and II cover amino acid residues 170 to 173 and 166 to 168, respectively. These regions are included in a putative binding site of the hydrocarbon moieties of the substrates which Brems *et al.* proposed for the chicken enzyme in an active site-directed photoaffinity labeling experiment (Fig. 2) (*13*). Thus, we introduced random mutations into these regions, expecting that the binding region of the hydrocarbon moieties of allylic substrates might contribute to the formation of intermediates and substrate specificity.

For the construction of each degenerate library, two PCR reactions were carried out using two sets of PCR primers. One of the four primers had a random sequence and all primers had a restriction enzyme site at the 5′-end. After digestion of the amplified fragments with suitable restriction enzymes, the two resulting fragments were simultaneously ligated back into the *Eco*065I and *Eco*RV sites of the original pHK119 plasmid. *E. coli* DH5$\alpha$ cells were then transformed with the plasmids. Libraries I and II have the potential to code for 16,000 (2×20×20×20) and 6,000 (15×20×20) amino acid sequences, respectively.

*In Vivo Analysis of Mutated Enzymes Derived from Library I and Determination of Their Sequences*—Forty plasmids were isolated from the clones that were randomly selected from library I. Cells of *E. coli* DH5$\alpha$/pACYC-IB were transformed with each plasmid, and after 3 days the color of the colonies was examined. Plasmid pACYC-IB encodes two genes, *i.e.* those of phytoene synthase (*crtB*) and phytoene desaturase (*crtI*). If GGPP synthase activity is expressed in *E. coli* cells transformed with the plasmid, the transformant should produce lycopene and become red. On the other hand, cells that do not exhibit GGPP synthase activity form white colonies. As a result, eight clones gave white colonies. The remaining clones formed red colonies, indicating that the enzymes encoded by the mutated GGPP synthase genes still exhibited GGPP synthase activity. The DNA sequences of the coding regions were determined. There is a frameshift in every gene derived from the white colonies. In the red colonies, random substitutions of amino acids between 170 and 173 were introduced as expected (Table I). Mutant I-25 contained 3 extra nucleic acids, thereby encoding a mutated GGPP synthase one amino acid

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                                                    II    I
GGPP Synthase 149-GQAVDMEFED--RIDI---KEQEYLDMISRKTA
                  ** .*.   .   ..*.   .*. *  .... ***
FPP Synthase  184-GQMLDLITAPVSKVDLSHFSEERYKAIVKYKTA
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Fig. 2. **Amino acid sequence alignment of the putative substrate binding regions of avian FPP synthase and *S. acidocaldarius* GGPP synthase.** The mutated regions of *S. acidocaldarius* GGPP synthase are boxed. The sequence of the fragment obtained in the photoaffinity experiment with avian FPP synthase is underlined, and the highly labeled region is boxed (*13*). The numbers to the left of the sequences indicate the position of the first amino acid displayed. * indicates an identified amino acid, · indicates a conserved amino acid.

longer than the wild type. These data suggest that a mutation in region I does not cause a complete loss of GGPP synthase activity.

*In Vitro Prenyltransferase Activities of Mutated GGPP Synthases Derived from Library I*—The 15 clones of *E. coli* DH5$\alpha$ containing mutated GGPP synthase genes, which formed red colonies, were cultured, and crude enzyme solutions were then prepared from the cultures. Since the *S. acidocaldarius* GGPP synthase is thermostable (*4*), the cell homogenates were heated at 55°C for 120 min prior to the enzyme assay to distinguish the thermostable GGPP synthase from thermolabile prenyltransferases derived from the host cells. Almost all the mutated GGPP synthases remained in the supernatant after heat treatment although many proteins derived from the host cells were precipitated (data not shown). These observations indicate that all mutated GGPP synthases were still thermostable. The prenyltransferase activities of the heat-treated en-

TABLE I. **DNA and amino acid sequences and GGPP synthase activities of mutated GGPP synthases derived from red colonies of library I.** Enzyme activity was determined as the radioactivity of 1-butanol extractable materials after a reaction involving 0.46 $\mu$M [1-$^{14}$C]IPP (2.00 GBq/mmol), 25 $\mu$M FPP, and 5 $\mu$g of heat-treated lysate as described under "EXPERIMENTAL PROCEDURES." The enzyme activity is expressed as relative activity, which is based on that of the wild type enzyme (3.15 nmol·min$^{-1}$·mg$^{-1}$).

| Mutant | DNA sequence | Amino acid sequence | Enzyme activity (%) |
|---|---|---|---|
| Wild type | GACATGATCTCA | Asp-Met-Ile-Ser | 100 |
| I-1 | GAGGGTGGGCGG | Glu-Gly-Gly-Arg | 6.64 |
| I-7 | GACTCCCGGTGC | Asp-Ser-Arg-Cys | 0.369 |
| I-8 | GATCGGGAAGGC | Asp-Arg-Glu-Gly | 2.02 |
| I-9 | GAGCCGGCGATG | Glu-Pro-Ala-Met | 1.84 |
| I-10 | GACTGTATGAAA | Asp-Cys-Met-Lys | 11.2 |
| I-11 | GAACCCCGCGTC | Glu-Pro-Arg-Val | 4.28 |
| I-15 | GACTGCGTTCGT | Asp-Cys-Val-Arg | 30.7 |
| I-21 | GATGCCGGTTAT | Asp-Ala-Gly-Tyr | 10.4 |
| I-23 | GAACCTGCGAGG | Glu-Pro-Ala-Arg | 70.6 |
| I-25 | GATGAGCTTTATGCG | Asp-Glu-Leu-Tyr-Ala | 49.3 |
| I-28 | GATTGGAACTTT | Asp-Trp-Asn-Phe | 5.63 |
| I-31 | GATGTGATGGTG | Asp-Val-Met-Val | 70.5 |
| I-34 | GAGTCGGCCTGG | Glu-Ser-Ala-Trp | 54.6 |
| I-36 | GATTGTGATGCC | Asp-Cys-Asp-Ala | 153 |
| I-40 | GATCGTCCTCGA | Asp-Arg-Pro-Arg | 30.9 |

TABLE II. **DNA and amino acid sequences and GGPP synthase activities of mutated GGPP synthases derived from red colonies of library II.** Enzyme activity was determined as the radioactivity of 1-butanol extractable materials after a reaction involving 0.46 $\mu$M [1-$^{14}$C]IPP (2.00 GBq/mmol), 25 $\mu$M FPP, and 5 $\mu$g of heat-treated lysate as described under "EXPERIMENTAL PROCEDURES." The enzyme activity is expressed as relative activity, which is based on that of the wild type enzyme (3.15 nmol·min$^{-1}$·mg$^{-1}$).

| Mutant | DNA sequence | Amino acid sequence | Enzyme activity (%) |
|---|---|---|---|
| Wild type | CAGGAATAC | Gln-Leu-Tyr | 100 |
| II-7 | GACCTGGTT | Asp-Leu-Val | 2.02 |
| II-9 | AGAAAAATT | Arg-Lys-Ile | 78.6 |
| II-10 | CCGTGCAGT | Pro-Cys-Ser | 51.1 |
| II-12 | TGTCAATCT | Cys-Gln-Ser | 65.2 |
| II-13 | AAATTCATT | Lys-Phe-Ile | 85.1 |
| II-14 | GTGAATGTT | Val-Asn-Val | 76.9 |
| II-19 | GTGAGTGAT | Val-Ser-Asp | 89.9 |
| II-21 | AAAGGTTAT | Lys-Gly-Tyr | 84.1 |
| II-27 | CCTAAATCT | Pro-Lys-Ser | 49.8 |
| II-28 | TAGGATGTT | Gln[a]-Asp-Val | 10.1 |
| II-29 | GATAAAGTT | Asp-Lys-Val | 60.2 |
| II-35 | GTACGGGTT | Val-Arg-Val | 58.5 |
| II-36 | AGGTCGTGT | Arg-Ser-Cys | 60.4 |
| II-37 | ATGATGGTT | Met-Met-Val | 67.7 |
| II-38 | CGCTAGAGT | Arg-Gln[a]-Ser | 6.68 |
| II-39 | ATAGAATGT | Ile-Glu-Cys | 45.7 |
| II-42 | CGTGGCAAT | Arg-Gly-Asn | 5.55 |
| II-45 | GGGTTATTT | Gly-Leu-Phe | 12.9 |
| II-48 | GAGGACGTT | Glu-Asp-Val | 68.7 |
| II-49 | GTGAGTTAT | Val-Ser-Tyr | 64.1 |
| II-79 | TTCCTATAT | Phe-Leu-Tyr | 70.4 |
| II-82 | TAGAACGCT | Gln[a]-Asn-Ala | 7.05 |
| II-99 | CTTAAAAGT | Leu-Lys-Ser | 90.0 |
| II-101 | CCGAGGTAT | Pro-Arg-Tyr | 63.0 |
| II-121 | TGTGGGGCT | Cys-Gly-Ala | 57.6 |
| II-125 | AAACTTTGT | Lys-Leu-Cys | 60.0 |
| II-126 | GGTTAGATT | Gly-Gln[a]-Ile | 18.6 |
| II-137 | TTACCTGGT | Leu-Pro-Gly | 16.9 |
| II-139 | AGATAGACT | Arg-Gln[a]-Thr | 16.0 |
| II-140 | GGGTATATT | Gly-Tyr-Ile | 44.9 |
| II-148 | ATGCATAAT | Met-His-Asn | 80.7 |
| II-149 | TATGATAAT | Tyr-Asp-Asn | 58.2 |

[a]Suppression of TAG codon.

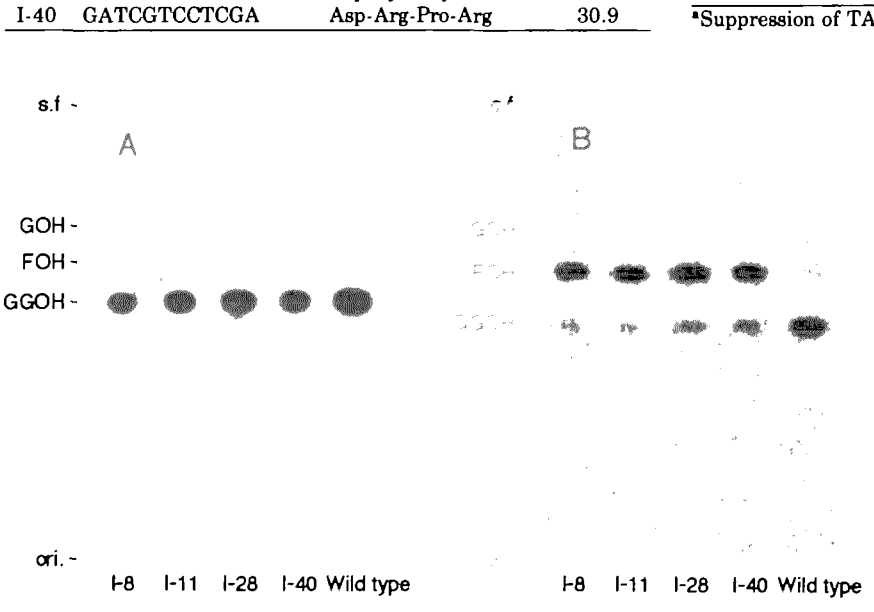


Fig. 3. **TLC-autoradiochromatograms of the alcohols obtained by enzymatic hydrolysis of the products formed by the mutated GGPP synthases from library I.** The sample obtained on incubation of 0.46 $\mu$M [1-$^{14}$C]IPP (2.00 GBq/mmol) and 25 $\mu$M FPP (A) or GPP (B) with the indicated enzyme was analyzed by reversed phase LKC-18 TLC as described under "EXPERIMENTAL PROCEDURES." Authentic standard alcohols: GOH, geraniol; FOH, (all-*E*)-farnesol; GGOH, (all-*E*)-geranylgeraniol. ori., origin; s.f., solvent front.

zymes were determined by measurement of the radioactivity of 1-butanol extractable materials after a reaction involving GPP or FPP as the primer substrate. All the activities observed in these experiments for mutated GGPP synthases could be fully ascribed to the enzymes because the heat-treated lysate of *E. coli* cells without transformation did not show any prenyltransferase activity. About two-thirds of the mutated enzymes showed weaker activity than the wild type enzyme when 25 $\mu$M FPP and 0.46 $\mu$M [1-$^{14}$C]IPP were used as substrates (Table I), although the expression levels of the enzymes were almost the same as that of the wild type enzyme (data not shown). These data suggest that the GGPP formation activities of these mutated enzymes under these conditions are weak. In order to confirm this, the products were analyzed by reversed-phase thin-layer chromatography. When the enzyme reactions were carried out using 25 $\mu$M GPP and 0.46 $\mu$M IPP, all the mutated GGPP synthases that showed much weaker activity than the wild type produced considerable amounts of FPP as an intermediate, the amount of GGPP formed being quite small. For example, under these conditions, the GGPP formation activity of

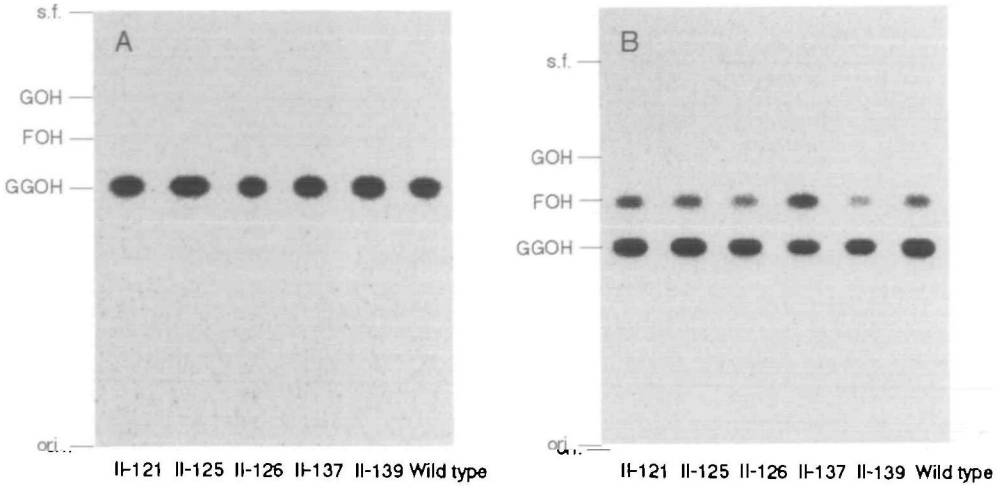


**Fig. 4. TLC-autoradiochromatograms of the alcohols obtained on enzymatic hydrolysis of the products formed by the mutated GGPP synthases from library II.** The sample obtained on incubation of 0.46 $\mu$M [1-$^{14}$C]IPP (2.00 GBq/mmol) and 25 $\mu$M FPP (A) or GPP (B) with the indicated enzyme was analyzed by reversed phase LKC-18 TLC as described under "EXPERIMENTAL PROCEDURES." Authentic standard alcohols: GOH, geraniol; FOH, (all-*E*)-farnesol; GGOH, (all-*E*)-geranylgeraniol. ori., origin; s.f., solvent front.

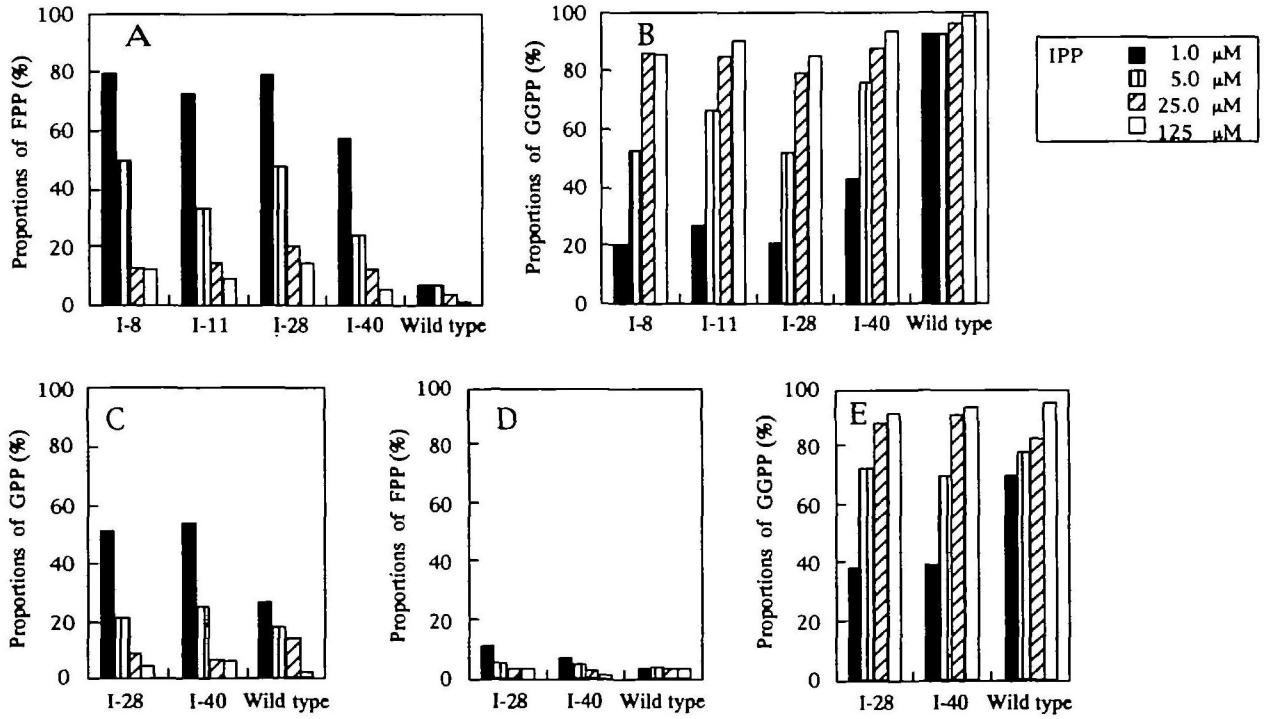


**Fig. 5. Effect of the IPP concentration on intermediate formation.** Panels A and B: The reaction products obtained on incubation of 25 $\mu$M GPP and the indicated amount of [1-$^{14}$C]IPP with the indicated pure enzyme were hydroxylated and then analyzed by reversed phase LKC-18 TLC as described under "EXPERIMENTAL PROCEDURES." The proportions of FPP (A) and GGPP (B) among the products were determined. Panels C, D, and E: The reaction products obtained on incubation of 25 $\mu$M DMAPP and the indicated amount of [1-$^{14}$C]IPP with the indicated enzyme were hydroxylated and then analyzed by reversed phase LKC-18 TLC as described under "EXPERIMENTAL PROCEDURES." The proportions of GPP (C), FPP (D), and GGPP (E) among the products were determined.

I-8 was about 1% that of the wild type enzyme. Some of the data are shown in Fig. 3B. When FPP was used as an allylic substrate, only GGPP was produced in all cases (Fig. 3A).

*Analysis of Mutated Enzymes Derived from Library II*—In the case of library II, 149 clones were examined for lycopene formation. Eight clones formed white colonies, but the genes contained a stop codon or a frameshift in the mutated region. The remaining clones formed red colonies. The prenyltransferase activities of the 32 mutated GGPP synthases derived from the red colonies of library II were measured by the same method as that for library I. Most of the enzymes showed as strong prenyltransferase activity as the wild type enzyme, but some enzymes showed lower activity (Table II). Some of the lower activities seem to reflect the expression levels of the mutated GGPP synthases, whose complete translation requires TAG suppression. Next, product analyses were carried out, some results of which are shown in Fig. 4, because almost all the mutated enzymes showed product distributions similar to that of the wild type. When FPP was used as the allylic substrate, GGPP was exclusively produced in every case (Fig. 4A). When GPP was used, GGPP was mainly produced, with significant formation of FPP (Fig. 4B). These data clearly indicate that region II is not directly involved in the catalytic activity or the formation of intermediates.

*Effect of the Substrate Concentration on the Product Distribution*—We thought that the formation of intermediates was related to a decrease in the responsiveness of the mutated enzymes to the IPP-metal ion complex because Ohnuma *et al.* (7) found that the product distribution of solanesyl diphosphate synthase, which catalyzes the formation of polyprenyl diphosphate with $C_{45}$, changed depending on the concentrations of both IPP and magnesium ions, and that sufficient concentrations of IPP and metal ions were necessary to produce the ultimate product. In order to determine the effect of the substrate concentration on intermediate formation in the reactions catalyzed by the mutated GGPP synthases, we fused MBP to the four mutated GGPP synthases of I-8, I-11, I-28, and I-40, all of which produced large amounts of FPP, and the wild type enzyme, and enzymes were purified homogeneously on an amylose resin affinity column. The purity of each enzyme was confirmed by sodium dodecyl sulfate polyacrylamide electrophoresis (12.5%, data not shown). The enzymes that were completely digested with Factor Xa were used. First, we investigated the effect of the concentration of IPP on intermediate formation (Fig. 5). When 125 $\mu$M IPP and 25 $\mu$M GPP were used (Fig. 5, A and B), the four mutated enzymes mainly produced GGPP with a smaller amount of FPP as expected, and the amount of FPP dramatically increased as the concentration of IPP was decreased, suggesting an increase in $K_m$ for IPP. In the case of the wild type enzyme, the ratio of FPP to the total amount of products slightly changed depending on the IPP concentration used. When DMAPP was used, the relative amounts of GPP (Fig. 5C), FPP (Fig. 5D), and GGPP (Fig. 5E) formed in the reaction with I-28 or I-40 were also dependent on the concentration of IPP. As the concentration of IPP was

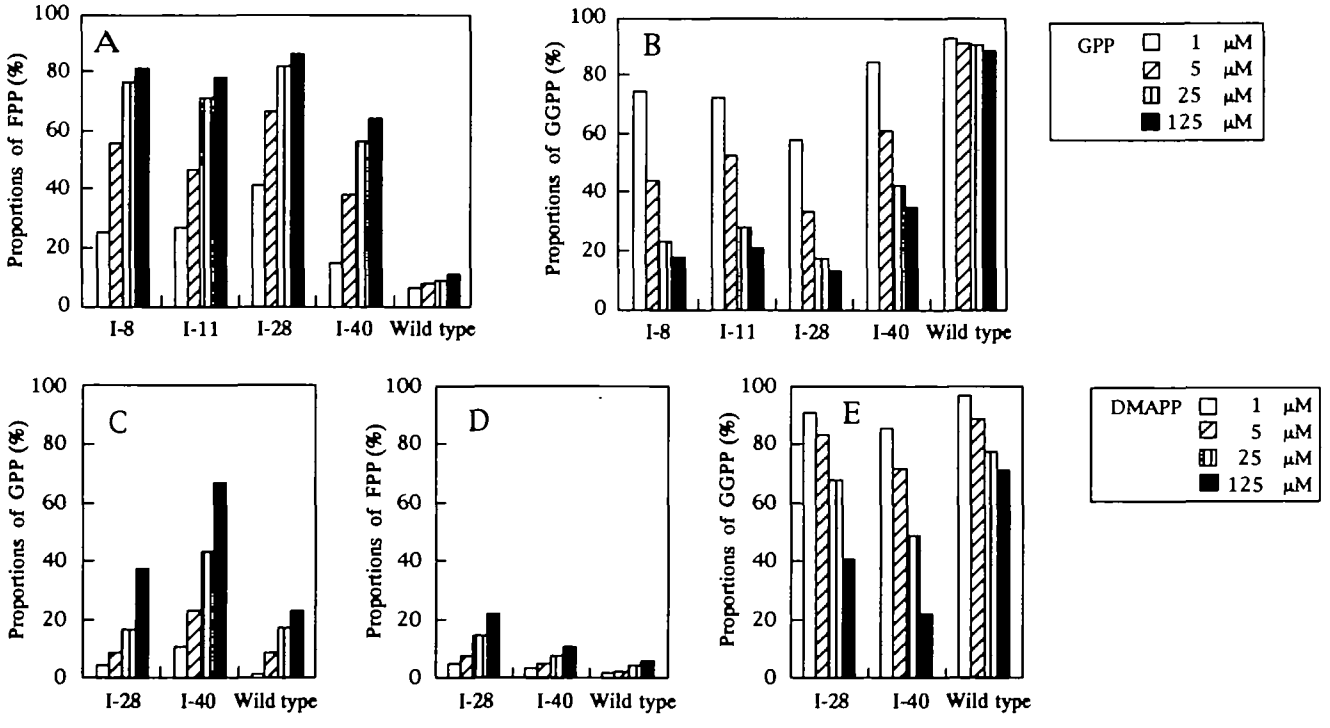


Fig. 6. **Effect of the concentration of allylic substrates on intermediate formation.** Panels A and B: The reaction products obtained on incubation of 1.0 $\mu$M [1-$^{14}$C]IPP and the indicated amount of GPP with the indicated pure enzyme were hydroxylated and then analyzed by reversed phase LKC-18 TLC as described under "EXPERIMENTAL PROCEDURES." The proportions of FPP (A) and GGPP (B) among the products were determined. Panels C, D, and E: The reaction products obtained on incubation of 1.0 $\mu$M [1-$^{14}$C]IPP and the indicated amount of DMAPP with the indicated enzyme were hydroxylated and then analyzed by reversed phase LKC-18 TLC as described under "EXPERIMENTAL PROCEDURES." The proportions of GPP (C), FPP (D), and GGPP (E) among the products were determined.

TABLE III. **Kinetic constants of the wild type and mutated GGPP synthases.** For the kinetic constants for allylic substrates, 25 $\mu$M [1-$^{14}$C]IPP, various amounts of an allylic substrate, and a purified enzyme were incubated as described under "EXPERIMENTAL PROCEDURES." For IPP, 25 $\mu$M FPP and various amounts of [1-$^{14}$C]IPP were used. Kinetic constants were calculated from the radioactivity of 1-butanol extractable materials.

| Mutant | DMAPP | | GPP | | FPP | | IPP | |
|---|---|---|---|---|---|---|---|---|
| | $K_m$ ($\mu$M) | $V_{max}$ (nmol·min$^{-1}$·mg protein$^{-1}$) | $K_m$ ($\mu$M) | $V_{max}$ (nmol·min$^{-1}$·mg protein$^{-1}$) | $K_m$ ($\mu$M) | $V_{max}$ (nmol·min$^{-1}$·mg protein$^{-1}$) | $K_m$ ($\mu$M) | $V_{max}$ (nmol·min$^{-1}$·mg protein$^{-1}$) |
| Wild type | 6.51 | 161 | 0.722 | 132 | 1.80 | 151 | 0.361 | 110 |
| I-8 | 17.7 | 72.1 | 2.01 | 136 | 0.247 | 139 | 5.99 | 111 |
| I-28 | 5.02 | 53.0 | 0.665 | 70.1 | 0.909 | 174 | 5.57 | 90.3 |
| I-40 | 10.4 | 72.3 | 1.41 | 784 | 1.11 | 217 | 2.58 | 155 |

increased, the formation of the intermediates, GPP and FPP, decreased so that GGPP became the major product. The change in GPP formation is much larger than that in FPP formation. The wild type enzyme also showed a similar dependency though it was less marked than that of the mutated enzymes.

Next, we examined the effect of the concentrations of the allylic substrates on product distribution. When GPP was used, the relative amount of FPP formed in the reaction by every mutated GGPP synthase dramatically changed depending on the concentration of GPP (Fig. 6, A and B). When 125 $\mu$M GPP was used, the major product was FPP, but GGPP was formed as the main product when 1 $\mu$M GPP was used. Moreover, the amount of FPP synthesized by the wild type hardly changed with the GPP concentration. In contrast, a change in intermediate formation depending on the concentration of DMAPP was observed with all enzymes including the wild type enzyme, and the dependency on the DMAPP concentration of the formation of GPP was greater than that of the formation of FPP (Fig. 6, C and D). Moreover, the amount of GPP was larger than that of FPP in almost all cases. These data suggest that, in the cases of both the mutated enzymes and the wild type enzyme, an allylic substrate competes with allylic intermediates for binding to the active site, and that an intermediate with a chain length near that of the substrate is easily expelled by the allylic substrate. The mutations in region I caused the enzymes to release intermediates more easily than the wild type. In any case, products longer than GGPP were never formed.

*Kinetic Constants of Mutated GGPP Synthases Derived from Library I*—The kinetic constants of the mutated GGPP synthases (I-8, 28, and 40) and the wild type enzyme for IPP, DMAPP, GPP, and FPP were determined by measurement of the radioactivity in the 1-butanol-extractable products (Table III). In these experiments, we used 25 $\mu$M of an allylic substrate and 25 $\mu$M IPP for determination of the kinetic constants for IPP and the allylic substrates, respectively. All mutated GGPP synthases showed increased $K_m$ values for IPP. For example, $K_{m(IPP)}$ of I-8 was 17 times larger than that of the wild type. The observation that these mutated GGPP synthases produced considerable amounts of intermediates, FPP and GPP, depending on the IPP concentration seems to certainly reflect the increased $K_m$ values for IPP. This is consistent with the previous observation for solanesyl diphosphate synthase (7). In the three mutants, I-8, I-28, and I-40, the $K_m$ values for DMAPP were increased and the $V_{max}$ values for DMAPP were decreased compared to those of the wild type, leading to the low activity toward DMAPP, whereas the activities toward other allylic substrates were not altered much. Although a significant sequence relationship of region I of the mutated GGPP synthases has not been found so far, these data indicate that the amino acids in region I play important roles in the recognition of both IPP and allylic substrates.

## DISCUSSION

We introduced random mutations into the two proximal regions, and analyzed their effects on catalytic activity, substrate specificity, and product specificity. Many mutated enzymes obtained from library I showed unique characteristics. In particular, an increase in the release of intermediates was prominent. The $K_m$ values for IPP of these mutants were greater than that of the wild type. Ohnuma *et al.* (7) and Matsuoka *et al.* (8) reported that intermediate product formation increased with decreasing IPP concentration. Therefore, the increased formation of intermediates reflects a deficiency of IPP for the mutants, which was due to the increase in $K_m$ for IPP. On the other hand, in region II, there is a high degree of flexibility as to the replacements of amino acids, and no significant change in catalytic activity or product specificity was observed. Therefore, region II seems to be unimportant.

What causes the change in $K_{m(IPP)}$ of the mutated GGPP synthases derived from library I? The detailed mechanisms and kinetic constants of prenyltransferases have been discussed only for the single condensation of GPP and IPP catalyzed by avian FPP synthase (*14, 15*). The FPP synthase has been shown to obey an ordered sequential mechanism for the synthesis of FPP from IPP and GPP. Thus, the equation is applied for the single condensation of FPP and IPP catalyzed by GGPP synthase because GGPP synthase can catalyze the same type of condensation as that by FPP synthase, and these enzymes have considerable conserved regions in their amino acid sequences. The steady state kinetic constants can be expressed in terms of individual rate constants as follows

$$E \underset{k_2}{\overset{k_1[FPP]}{\rightleftharpoons}} E\cdot FPP \underset{k_4}{\overset{k_3[IPP]}{\rightleftharpoons}} E\cdot FPP\cdot IPP \xrightarrow{k_5} E\cdot PP_i\cdot GGPP \xrightarrow{k_6} E \quad (1)$$

where

$$V_{max} = \frac{k_5 k_6 [Et]}{k_5 + k_6} \quad (2)$$

$$K_{m(FPP)} = \frac{k_5 k_6}{k_1 (k_5 + k_6)} \quad (3)$$

$$K_{m(IPP)} = \frac{k_6 (k_4 + k_5)}{k_3 (k_5 + k_6)} \quad (4)$$

As can be judged from Eq. 4, the increase in $K_{m(IPP)}$ is

caused not only by an increase in $K_{d(IPP)}$ but also by a decrease in $k_5$. At present, it is unclear which is more suitable. However, these findings suggest the possibility that mutated enzymes showing a decrease in condensation rate or a decrease in affinity for IPP show properties similar to these of I-8, 11, 28, and 40.

Although a change in product distribution depending on the IPP concentration has been reported so far, we found that the product distribution also depended on the allylic substrate. Though these phenomena are prominent for the mutated GGPP synthases, the wild type enzyme also showed a similar dependency (Fig. 6). In the case of the mutated enzymes, when GPP was used as the allylic substrate, the relative amount of FPP formed dramatically changed depending on the GPP concentration, whereas the relative amount of FPP did not change much with the DMAPP concentration. These results indicate that the allylic substrate effectively competes with the intermediate with a chain length near that of the allylic substrate so that the intermediate is released. A similar phenomenon was also observed in the case of dehydrodolichyl diphosphate synthase of rat (*8, 16*). This enzyme forms a significant amount of (2*Z*,6*E*,10*E*)-GGPP when FPP is used as the allylic substrate.

As shown in Table III, the mutated GGPP synthases of I-8 and 40 showed decreased activity toward DMAPP, which mainly results from the increased $K_m$ values for DMAPP and the decreased $V_{max}$ values for DMAPP. Moreover, I-9 and I-11 do not accept DMAPP. These data demonstrate that region I is responsible for the recognition of allylic substrates. Brems and Rilling (*17*) showed that the catalytic site, once photolabeled, loses its capacity to bind both substrates as well as its catalytic function. Therefore, these facts support the idea that the binding sites for IPP and an allylic substrate are situated near region I. The properties of substrate specificity are similar to those of GGPP synthases from cow (*1*) and yeast (*2*).

The crystal structure of avian FPP synthase was determined by Tarshis *et al.* (*18*). Avian FPP synthase, which exhibits modest amino acid similarity to *S. acidocaldarius* GGPP synthase, is composed of 13 $\alpha$-helices joined by connecting loops, 8 of which form a large central cavity. The active site of FPP synthase seems to be located in the large central cavity, and two aspartate-rich motifs, which are highly conserved among the prenyltransferase family and are thought to bind the diphosphate moieties of IPP and allylic substrates through magnesium ion bridges, are found in the helices composing the cavity. Region I is located in the helix that composes the cavity and exists in the middle of the two helices with the aspartate-rich motifs. It was reported that, in yeast FPP synthase, the mutation of lysine to glutamic acid at position 197, which is located in a conservative region of prenyltransferases and corresponds to position 175 of *S. acidocaldarius* GGPP synthase, changed the product specificity (*19-21*). The mutant produced a large amount of GPP as an intermediate, which is similar to observed for the mutants obtained from library I. The side chain of the lysine of avian FPP synthase extends toward the middle of the cavity and is located between two aspartate-rich motifs. These data might suggest that the lysine stabilizes the binding of both substrates and prevents the release of intermediates through ionic bonding. Our mutations might bring about a change in the side-chain angle of lysine, resulting in the release of the intermediate. Further analysis is necessary to elucidate the precise mechanism.

In any case, we could not find a mutant that produced polyprenyl diphosphates with prenyl chains longer than GGPP. Recently, we succeeded in converting both FPP synthase to GGPP synthase (*22*), and GGPP synthase to hexaprenyl diphosphate synthase (*23*) using random chemical mutagenesis and phenotypic screening methods. Based on the results of analysis of the mutated enzymes, we revealed that several amino acids contribute to the ultimate chain length and that, in particular, the fifth amino acid before the first aspartate rich motif is extremely important (unpublished results). However, none of them were situated around region I. Therefore, the amino acid residues around region I do not seem to contribute to the ultimate chain length, which is determined by prenyltransferases, but to the release of intermediates and substrate recognition.

Although we observed that *in vitro* GGPP formation dramatically decreased as the concentration of IPP decreased in some mutants derived from library I, the color of the cells that co-expressed the mutated GGPP synthase, phytoene synthase, and phytoene desaturase did not differ from that of the wild type GGPP synthase. These results indicate that the amount of GGPP in the cells transformed with the mutated GGPP synthases is sufficient for lycopene formation, because of a sufficient concentration of IPP for the mutated enzyme or a large amount of the mutated enzyme expressed. In fact, *ca.* 10 $\mu$mol·day$^{-1}$ of GGPP synthase activity was obtained for 1 g of wet cells of *E. coli* DH5$\alpha$/pHK119 when the assay was carried out at 37°C, and *ca.* 1 $\mu$mol of lycopene was formed with 1 g of wet cells of *E. coli* DH5$\alpha$/pACYC-IB, pHK119. These data indicate that the amount of GGPP synthase expressed in *E. coli* is too large for detection of the change in the mutant GGPP synthase activity observed in this study. Therefore, in order to detect mutant GGPP synthases using the red-white screening system, we must decrease the amount of a mutant GGPP synthase by decreasing the copy number of a plasmid or by using a promoter weaker than *lac*.

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