FEBS Letters 481 (2000) 68–72 FEBS 24085

# The role of histidine-114 of *Sulfolobus acidocaldarius* geranylgeranyl diphosphate synthase in chain-length determination

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Received 2 June 2000; accepted 13 July 2000

Edited by Richard Cogdell

**Abstract** ***Sulfolobus acidocaldarius*** **geranylgeranyl diphosphate synthase yields (all-*E*)-$C_{20}$ prenyl diphosphate as a final product. The three-dimensional model of the enzyme suggested that removing two bulky residues at 77 and 114 would allow additional prenyl-chain elongation. To test this, we examined several mutants with substitutions at 77 and/or 114. As a result, the mutants, F77G, F77G and H114A, F77G and H114G, H114A, and H114G gave $C_{30}$, $C_{45}$, $C_{50}$, $C_{30}$ and $C_{40}$ as the main long product, respectively. These observations indicate that histidine-114 plays a crucial role in chain-length determination along with phenylalanine-77.** 

*Key words:* Prenyltransferase; Geranylgeranyl diphosphate; Product specificity; Site-directed mutagenesis; Molecular modeling

## 1. Introduction

In archaea, geranylgeranyl diphosphate (GGPP, $C_{20}$) is a key precursor for ether-linked lipids, which constitute their cell membranes and seem to allow their survival under severe conditions, e.g. at high temperatures or extreme pHs [1,2]. GGPP is also regarded as a substrate for the biosynthesis of a diphytanylglyceryl-modified protein [3,4] and undecaprenol, which functions as a sugar-carrier lipid, as in bacteria (Hemmi et al., submitted for publication).

GGPP synthase (GGPS, EC 2.5.1.29) from a thermoacidophilic archaeon *Sulfolobus acidocaldarius* catalyzes the consecutive condensation of isopentenyl diphosphate (IPP, $C_5$) with dimethylallyl diphosphate (DMAPP, $C_5$), geranyl diphosphate (GPP, $C_{10}$), or farnesyl diphosphate (FPP, $C_{15}$) to give GGPP ($C_{20}$) as a final product [5,6]. Previous studies of our group and others have shown that, as for short-chain prenyl diphosphate synthases including *S. acidocaldarius* GGPS, the processes of the chain elongation and termination occur as follows. After binding of the diphosphate moiety of the allylic substrate via $Mg^{2+}$ to the aspartates of the first aspartate-rich motif (FARM), the prenyl chain extends into a hydrophobic pocket surrounded by α-helices in the enzyme during the consecutive condensation of IPP [7,8]. Then, the elongation is stopped precisely at a definite chain length by the direct interaction of the ω-terminus of the product with a bulky amino acid upstream from the FARM [9–11]. In the case of *S. acidocaldarius* GGPS, replacement of some bulky amino acids before the FARM with less bulky residues causes the expansion of the hydrophobic pocket to yield the products with a chain length longer than $C_{20}$ [12].

We have also previously reported that, concerning *S. acidocaldarius* GGPS and *Bacillus stearothermophilus* FPP synthase, some amino acids not situated upstream from the FARM affect the regulation of the product chain length [10,13]. However, it is still unclear how these amino acids take part in the chain-length determination.

Here, we built up a plausible three-dimensional model structure of *S. acidocaldarius* GGPS according to the structure of avian FPP synthase [7] and assigned the putative amino acids, which should hinder the chain elongation [12]. The spatial relationship of these amino acids in the model suggests that the prenyl chain proceeds through a bent path in the mutated GGPS with a deeper pocket. This model also gave us an idea that it might be possible to produce the longer prenyl chain efficiently if a straight path was created in the enzyme by replacing two amino acids, phenylalanine-77 and histidine-114, with smaller residues. To test this, we introduced the substitution of alanine or glycine at 77 and/or 114 and analyzed the products of the mutated enzymes.

Histidine-114 is assumed to lie on the same α-helix that phenylalanine-118 is located on. Our previous study showed that the mutated GGPS with F118L produced a small amount of $C_{25}$ [10]. In addition, regarding *B. stearothermophilus* FPP synthase, the mutation of valine-157, which is placed in the region corresponding to that including histidine-114 and phenylalanine-118 of *S. acidocaldarius* GGPS, changes the product specificity [13]. The data obtained in this study would be a clue to the role of these amino acids not situated before the FARM in the chain-length determination.

## 2. Materials and methods

### 2.1. Materials

[1-$^{14}$C]IPP was purchased from Amersham Pharmacia Biotech. Non-labeled IPP, DMAPP, GPP (all-*E*)-FPP and (all-*E*)-GGPP were presented by Dr. Ogura. A precoated reversed-phase thin-layer chromatography (TLC) plate, LKC18, and a high performance TLC plate, RP-18, were purchased from Whatman and Merck, respectively. A plasmid, pBS-GGPS, which encodes the wild-type GGPS, has been described in our previous paper [10].

### 2.2. Molecular modeling for GGPS

A three-dimensional model structure of *S. acidocaldarius* GGPS

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*Abbreviations:* GGPP, (all-*E*)-geranylgeranyl diphosphate; GGPS, geranylgeranyl diphosphate synthase; IPP, isopentenyl diphosphate; DMAPP, dimethylallyl diphosphate; GPP, geranyl diphosphate; FPP, (all-*E*)-farnesyl diphosphate; FARM, first aspartate-rich motif; TLC, thin-layer chromatography

0014-5793/00/$20.00 
PII: S0014-5793(00)01972-4

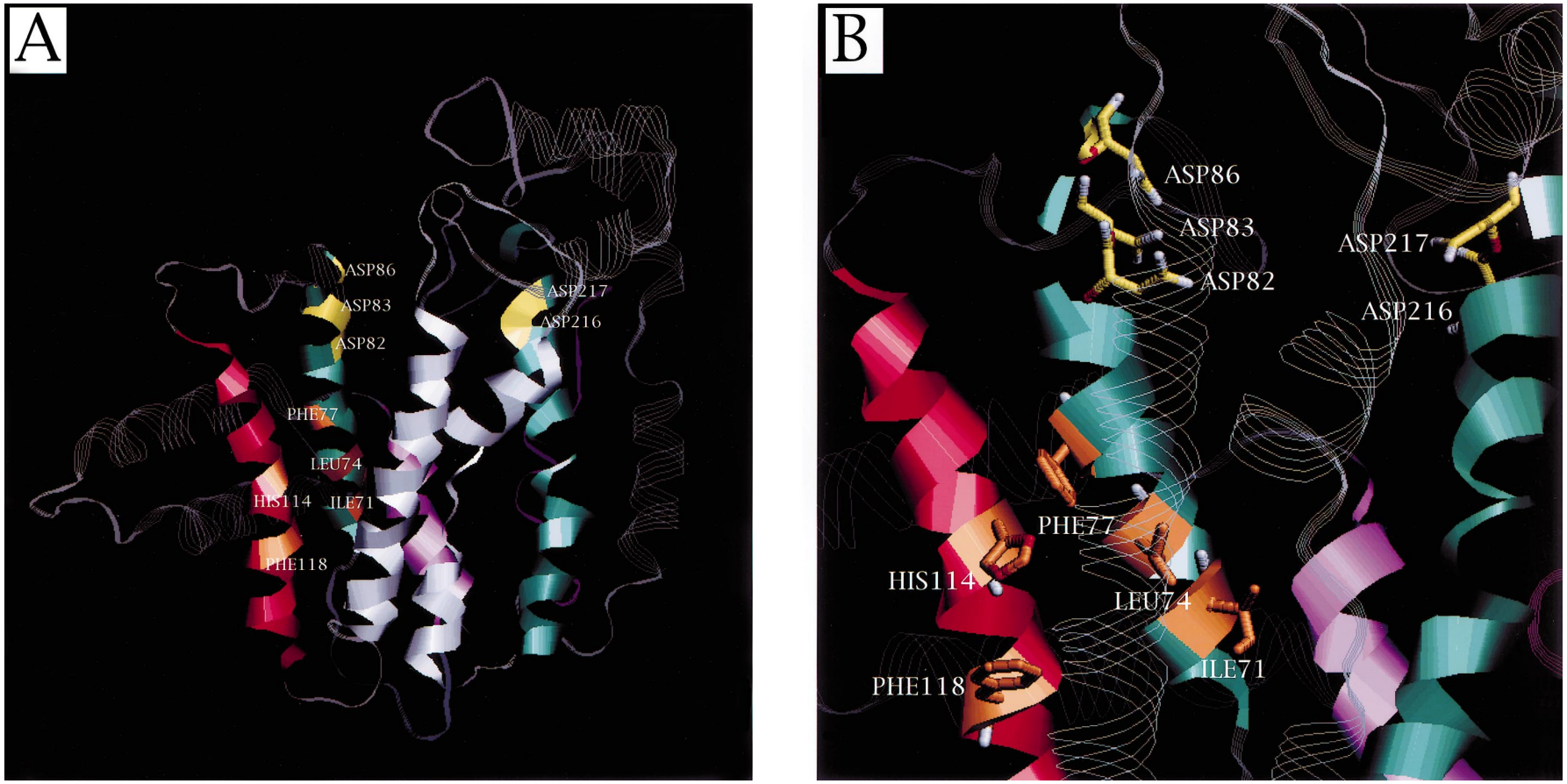


Fig. 1. Calculated three-dimensional structure of *S. acidocaldarius* GGPS. A: The overall model structure calculated based on a structure of avian FPP synthase [7]. Two aspartate-rich motifs are indicated by the aspartate residues as yellow ribbons. The amino acids responsible for the chain-length determination are indicated as orange ribbons. Histidine-114 lies in the α-helix indicated as a red ribbon. The six α-helices indicated as green, white and violet ribbons, respectively, are assumed to form the cavity into which the prenyl chain elongates. B: Magnified view of the presumed catalytic domain. Two aspartate-rich motifs are indicated by the aspartate residues as yellow sticks. The amino acids responsible for the chain-length determination are indicated as orange sticks. Atoms shown as white and red in color represent those of oxygen and nitrogen, respectively.

was built based on the structure of avian FPP synthase [7], using the Insight II Homology software (Biosym Technologies, San Diego, CA, USA) [14]. The model was then subjected to limited energy refinement with the Insight II Discover 3 software (Biosym Technologies, San Diego, CA, USA).

### 2.3. Construction of mutated GGPSs

Site-directed mutagenesis was carried out by the method of Kunkel [15]. Plasmids, pBS-GGPS-L15, pBS-GGPS-L18 and pBS-GGPS-L19, were constructed using a single strand DNA of pBS-GGPS and oligonucleotides: oligo-F77G, 5′-CATACT**GG**TACGCTTGT**TC**ATG-ATG-3′; oligo-H114A, 5′-ATATTAGCTGGAGATCTACTA**GC**TG-CAAAGGCT-3′; oligo-H114G, 5′-ATATTAGCTGGAGATCTAC-TA**GG**TGCAAAGGCT-3′. A mismatch is indicated by a bold letter, and a newly introduced restriction site is underlined. A single strand DNA of pBS-GGPS-L15 was employed for additional mutagenesis using oligo-H114A and oligo-H114G as a primer to construct plasmids, pBS-GGPS-L16 and pBS-GGPS-L17, respectively. All mutations were confirmed by DNA sequencing.

### 2.4. Preparation of the wild-type and mutated GGPSs

*Escherichia coli* XL1-Blue was transformed with each of the plasmids described above, and the wild-type and mutated enzymes were prepared according to the method reported previously [12].

### 2.5. Measurement of enzymatic activity and product analysis

The assay mixture contained, in a total volume of 200 μl, 10 mM phosphate buffer, pH 5.8, 5 mM $MgCl_2$, 0.1% Triton X-100, 25 nmol of [1-$^{14}$C]IPP (37 GBq/mol), 25 nmol of an indicated allylic substrate and a suitable amount of the wild-type or mutated enzyme. The incubation was carried out at 55°C for 30 min and stopped by chilling quickly. The mixture was shaken with 1 ml of 1-butanol saturated with $H_2O$ to extract the products of prenyl diphosphate synthase. The 1-butanol extract was washed with water saturated with NaCl and the radioactivity in the 1-butanol extract was measured with a liquid scintillation counter. The chain lengths and amounts of the products were determined by the TLC analysis as described previously [12]. For the determination of the length of some minor products, which have an extremely long prenyl chain ($>C_{60}$), the assay mixture was scaled up by a factor of ten.

## 3. Results and discussion

### 3.1. Molecular modeling for S. acidocaldarius GGPS

We have succeeded in purifying *S. acidocaldarius* GGPS to homogeneity (Kato et al., unpublished results). However, our attempt to crystallize the protein for the determination of its tertiary structure has been so far unsuccessful. Thus, in order to obtain the information on the structure participating in the product specificity of GGPS, we have tried to build up a three-dimensional model of this enzyme (Fig. 1) based on the structure of avian FPP synthase determined by Tarshis et al. [7]. *S. acidocaldarius* GGPS and avian FPP synthase catalyze similar condensations and show a considerable similarity in the primary structure (41%). According to this calculated model, two aspartates in the FARM, around which the condensation reaction occurs [5,8], and three bulky amino acids, phenylalanine-77, leucine-74 and isoleucine-71, which were found to play an important role in stopping the prenyl-chain elongation [12], lie on a bent line (Fig. 1B). The mutated GGPS with the replacement of these bulky amino acids by glycines yielded $C_{40}$ as the main long product [12]. Considering the model structure of GGPS, it was assumed that, in the mutated enzyme, the prenyl chain proceeds through a bent path created by the elimination of these bulky residues. In addition, the GGPS model depicted a straight line with the amino acids of two aspartates in the FARM, phenylalanine-77, and 'histidine-114' (Fig. 1B). The spatial relationship of these amino acids brought us the prospect that removing the bulky residues at positions 77 and 114 would create a straight path in the enzyme for prenyl-chain stretching and would enable the efficient production of a long prenyl chain. To test this possibility, we introduced substitutions of alanine or glycine at 77 and/or 114 and analyzed the products of the mutated enzymes.

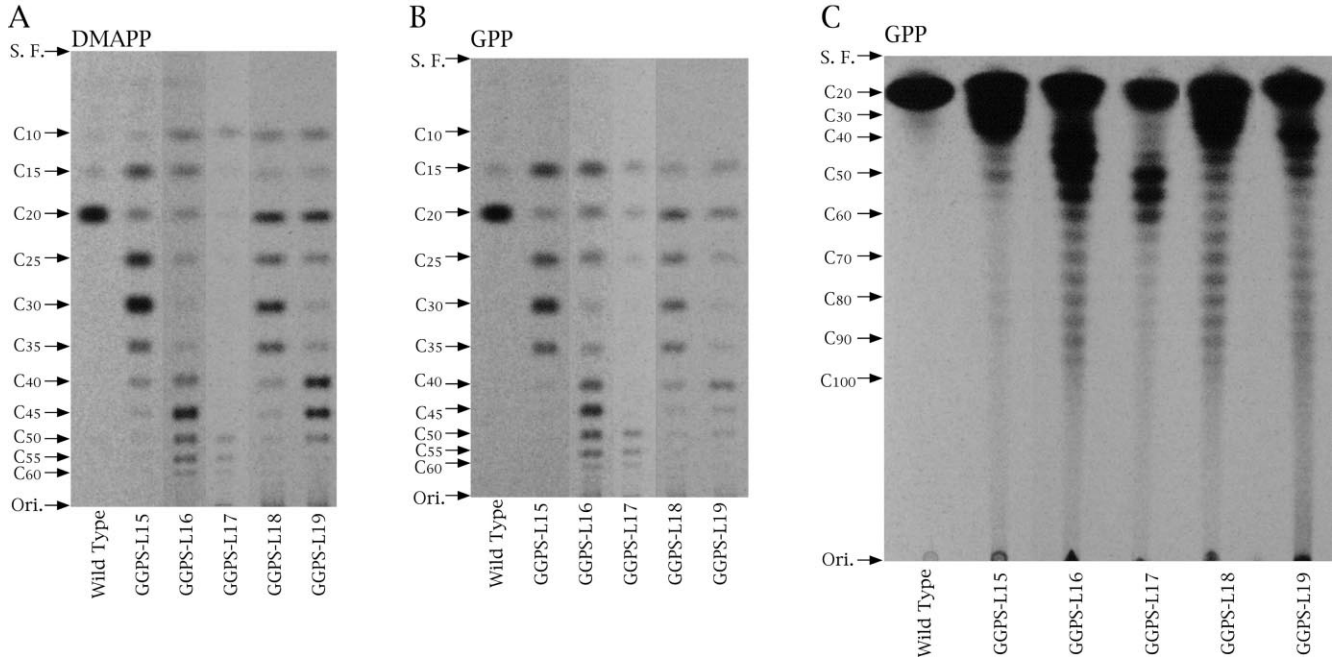


Fig. 2. TLC-autoradiochromatograms of the prenyl alcohols obtained by the hydrolysis of the reaction products with the wild-type and the mutated GGPSs. The sample obtained by incubation of [1-$^{14}$C]IPP and DMAPP (A), or GPP (B, C) with the indicated enzyme was analyzed by reversed phase LKC18 TLC (A, B) or high performance RP-18 TLC (C). Ori., origin; S.F., solvent front.

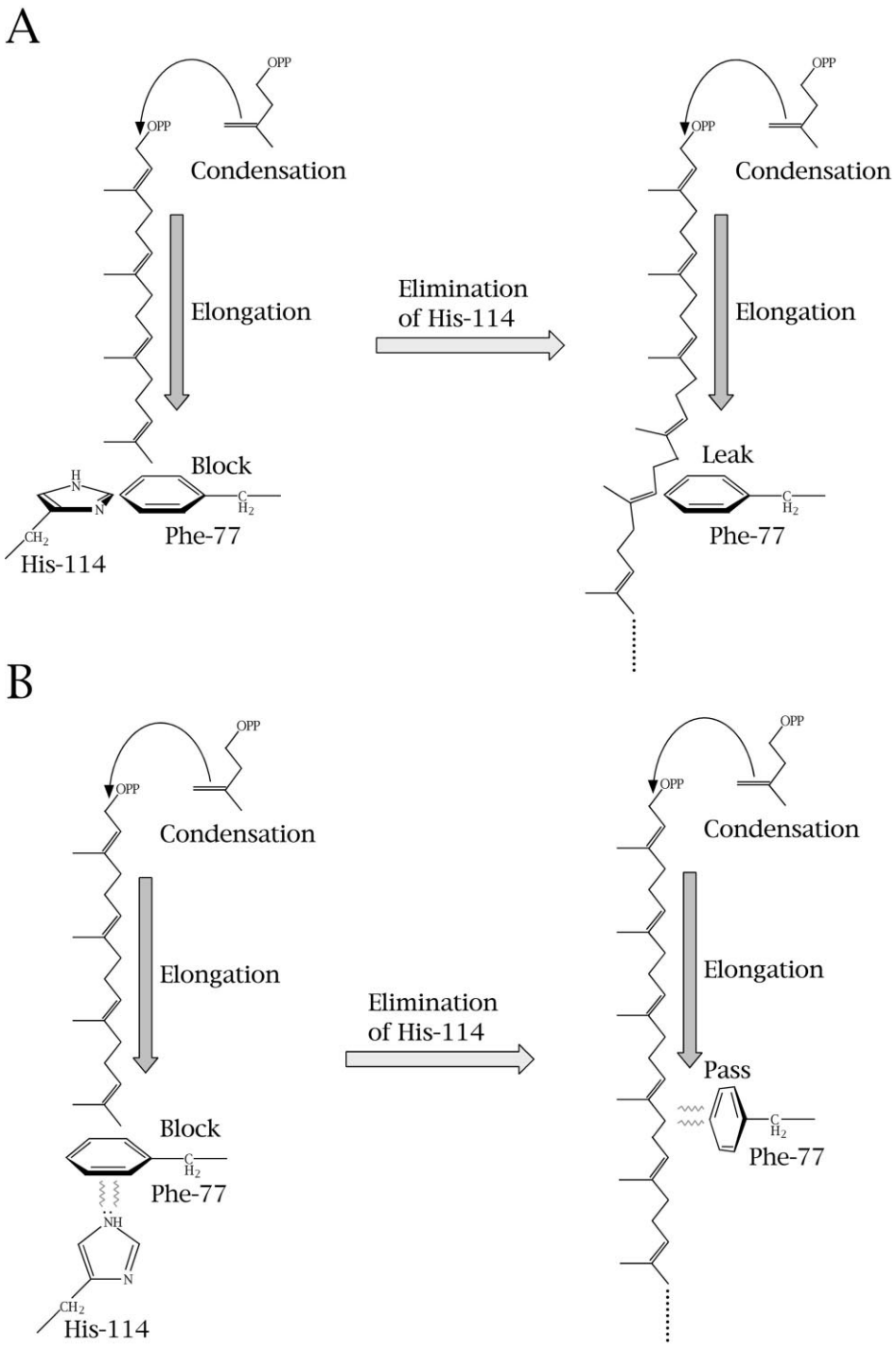


Fig. 3. Two possible roles that histidine-114 plays in the chain-length determination of *S. acidocaldarius* GGPS. A: A bottom model. B: A basis model.

### 3.2. Measurement of the activity of mutated GGPSs

All mutated GGPSs showed a specific activity comparable to that of the wild-type enzyme, except for GGPS-L17 (F77G and H114G) (Table 1). Although its activity was approximately 2–5% of the wild-type, this double-mutated enzyme exhibited a detectable activity and a thermostability to resist 15 h of heat treatment at 55°C.

### 3.3. Product analysis of mutated GGPSs

The reaction products with these mutated enzymes were analyzed by reversed-phase TLC after hydrolysis of a diphosphate moiety by acid phosphatase. When the enzymatic reaction was carried out using 125 μM [1-$^{14}$C]IPP and 125 μM DMAPP, the wild-type GGPS produced mostly $C_{20}$ (molar ratio, 87%) and never gave products whose chain lengths were longer than $C_{20}$ as reported previously [10]. In contrast, GGPS-L15 (F77G) yielded $C_{30}$ as a main product (33%), and GGPS-L16 (F77G and H114A) and GGPS-L17 (F77G and H114G) gave $C_{45}$ (15%) and $C_{50}$ (4.6%) mainly in the long-chain region ($> C_{20}$), respectively (Fig. 2A). This result sug-

Table 1
Specific activity of the wild-type and the mutated GGPSs

| Enzyme | Specific activity (nmol/min/mg) | | | |
|---|---|---|---|---|
| | DMAPP | GPP | FPP | GGPP |
| GGPS ($C_{20}$, wild-type) | 6.7 | 5.4 | 1.8 | ND[a] |
| GGPS-L15 ($C_{30}$, F77G) | 8.7 | 3.1 | 2.3 | 0.15 |
| GGPS-L16 ($C_{45}$, F77G and H114A) | 1.3 | 0.67 | 0.55 | 0.056 |
| GGPS-L17 ($C_{50}$, F77G and H114G) | 0.12 | 0.11 | 0.083 | 0.039 |
| GGPS-L18 ($C_{30}$, H114A) | 3.4 | 1.4 | 2.0 | 0.14 |
| GGPS-L19 ($C_{40}$, H114G) | 4.3 | 0.94 | 0.65 | 0.19 |

Each name of an enzyme is shown with the carbon number of the main long-chain product and the amino-acid replacement(s) in parentheses. Enzymatic activities were determined by measuring the amount of [1-$^{14}$C]IPP incorporated into 1-butanol extractable materials when 125 μM of [1-$^{14}$C]IPP and 125 μM of the indicated primer substrate were used.
[a]ND, not determined.

gests that the prenyl chain elongates through a straight path in the enzyme molecule formed by the elimination of the bulky residues at positions 77 and 114, and that the extended path of the double-mutated enzymes, GGPS-L16 and GGPS-L17, is longer than that of GGPS-L15, into which the single mutation was introduced.

It is interesting that the single-mutated enzymes, GGPS-L18 (H114A) and GGPS-L19 (H114G), which were assumed to have a similar path to that of the wild-type, mainly gave $C_{30}$ (15%) and $C_{40}$ (10%), respectively, regarding the products over $C_{20}$ (Fig. 2A). Similar product distributions were observed when GPP, FPP or GGPP was used as the allylic substrate for all the mutated enzymes (Fig. 2B).

As the mutated GGPSs can produce extremely long-chain polyprenyl diphosphates, we analyzed the precise lengths of the products with a high performance TLC system (Fig. 2C). As a result, the longest products with GGPS-L15, GGPS-L16, GGPS-L17, GGPS-L18 and GGPS-L19 were found to be $C_{85}$, $C_{100}$, $C_{90}$, $C_{100}$ and $C_{100}$, respectively. The hydrocarbon of these extremely long products seems to have protruded from the span of the path in the enzymes during the reaction.

### 3.4. The role of histidine-114 of GGPS in the chain-length determination

As described in Section 3.3, the single-mutated enzymes, GGPS-L18 and GGPS-L19, unexpectedly yielded products longer than $C_{20}$. Two models are proposed to explain these results. One is that histidine-114 of the wild-type GGPS forms a part of the bottom of the reaction pocket as well as phenylalanine-77 (Fig. 3A). The other is that histidine-114 immobilizes a broad face of the aromatic ring of phenylalanine-77 against the prenyl chain growing into the hydrophobic pocket. If this model is correct, in the single-mutated enzyme lacking the histidine at 114, the aromatic ring might turn around to make some passage for the prenyl-chain stretching (Fig. 3B). This 'basis model' is supported by the calculated structure of GGPS (Fig. 1B). GGPS-L18 (H114A) and GGPS-L19 (H114G) showed an increased production of $C_{20}$ compared to that of GGPS-L15 (F77G) (Fig. 2A,B). This observation suggests that a portion of the products interact with phenylalanine-77 when they reach at $C_{20}$, which agrees with both of the above-mentioned models.

Although we cannot figure out the precise role of the histidine-114, the results in this paper provide clear evidence that histidine-114 of *S. acidocaldarius* GGPS is important for the formation of the hydrophobic cavity to accommodate hydrocarbon of the product. Phenylalanine-118, which was previously shown to influence the regulation of the product chain length [10], might interact with histidine-114 and thereby participate in the mechanism of the chain-length determination (Fig. 1B). For a detailed study of the function of histidine-114, together with phenylalanine-118, it is indispensable to determine the crystal structure of *S. acidocaldarius* GGPS, and thus, further efforts are now in progress.

*Acknowledgements:* We are grateful to Dr. K. Ogura for providing us allylic substrates. We thank Dr. T. Nakayama, Dr. S. Ohnuma and Dr. T. Koyama, for their critical reading of this manuscript. This work was supported in part by grants-in-aid for Scientific Research from the Ministry of Education, Science and Culture of Japan. K.H. is a recipient of Research Fellowships of the Japan Society for the Promotion of Science for Young Scientists.

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