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Recognition of Allylic Substrates in *Sulfolobus acidocaldarius* Geranylgeranyl Diphosphate Synthase: Analysis Using Mutated Enzymes and Artificial Allylic Substrates[1]

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We examined the substrate specificity of two mutated geranylgeranyl diphosphate synthases, I-9 and I-11, with respect to several artificial substrates. These mutated enzymes have replacements in the amino acid sequences from positions 170 to 173, which are thought to be a part of the putative substrate binding region. The wild-type enzyme catalyzes the condensation of IPP with a series of (2*E*)-3-methyl-2-alkenyl diphosphates to give products with carbon numbers between 14 and 21. On the other hand, the mutated enzymes show lower activities for artificial substrates with short alkyl chains than those of the wild-type enzyme though the carbon numbers of the products are similar to those in the case of the wild-type. The mutated enzyme I-11 never accepts artificial substrates shorter than C_8. Analysis of additional mutated enzymes revealed that the characteristics of the mutated enzymes arise from a few substitutions within positions 171 to 173. These results indicate that the amino acids in the positions 171 to173 of the geranylgeranyl diphosphate synthase from *Sulfolobus acidocaldarius* are involved in recognition of short allylic substrates, such as dimethylallyl diphosphate, but not in recognition of the chain length of the products.

Key words: artificial substrates, enzyme mechanism, geranylgeranyl diphosphate synthase, isoprenoids, prenyltransferase.

Geranylgeranyl diphosphate (GGPP) synthase, which is a member of the prenyltransferase family, catalyzes the condensation of isopentenyl diphosphate (IPP) with a few allylic substrates to form an amphiphilic product, GGPP, which has four isoprene units (Fig. 1). The GGPP synthase has been found in a number of organisms, and its product, GGPP, works as a key intermediate in biosyntheses of carotenoids, geranylgeranylated proteins, chlorophylls, diterpenes, and archaeal ether-linked lipids. Although all GGPP synthases produce GGPP as the ultimate product, their substrate specificities and intermediate accumulations show marked variations. GGPP synthases from plants almost equally accept three allylic primer substrates, dimethylallyl diphosphate (DMAPP), geranyl diphosphate (GPP), and farnesyl diphosphate (FPP), to give GGPP (*1-3*), whereas those from animals (*4, 5*) and from fungi (*6, 7*) prefer FPP as an allylic substrate and show low activities for the short chain primers. Eubacterial GGPP synthases seem to have no common preference for allylic substrates. *Micrococcus luteus* GGPP synthase recognizes DMAPP and FPP, but not GPP (*8*). On the other hand, GGPP synthase of *Erwinia uredovora* accepts GPP and FPP, but not DMAPP as substrates (*9*). Moreover, archaeal GGPP synthase accumulates a considerable amount of FPP as an intermediate (*10-12*). The archaeal enzyme has been thought to be an ancestor of prenyltransferase (*13, 14*) and to play a bifunctional role in the synthesis of both FPP and GGPP *in vivo*.

Several genes of these GGPP synthases have been identified from different organisms, and homology search revealed that the sequences of these GGPP synthases are highly conserved (*13*). From analyses of mutated GGPP synthase, our group found that an aromatic amino acid at the fifth position before the first aspartate-rich consensus sequence determined the chain length of the final product, GGPP (*15*). However, amino acids that contribute to substrate specificity and intermediate accumulation have not been identified. Recently, during analyses on substrate specificity and intermediate accumulation using mutated GGPP synthases of archaea *Sulfolobus acidocaldarius*, we found two mutated GGPP synthases, I-9 and I-11, that showed a weak activity for the short chain primer, DMAPP (*16*). This specificity is similar to those observed in the enzymes from fungi and animals. In this study, we examined the

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Abbreviations: DMAPP, dimethylallyl diphosphate; FOH, farnesol; FPP, farnesyl diphosphate; GGOH, geranylgeraniol; GGPP, geranylgeranyl diphosphate; GPP, geranyl diphosphate; IPP, isopentenyl diphosphate.

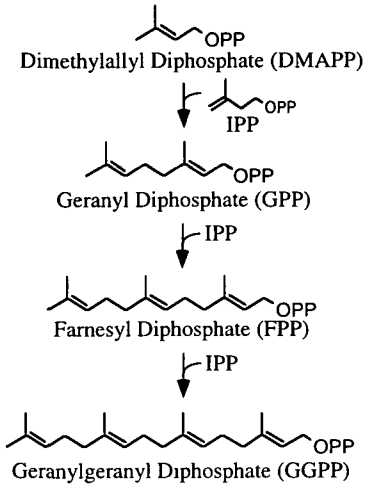


Fig. 1. **Consecutive reaction of GGPP synthase of *S. acidocaldarius*.**

substrate specificities of the mutated GGPP synthases using artificial allylic substrates.

EXPERIMENTAL PROCEDURES

Materials—Precoated reversed-phase thin layer chromatography plates, LKC-18 were purchased from Whatman Chemical Separation. (all-*E*)-FPP, (all-*E*)-GGPP, GPP, and DMAPP were the same preparations as used in the previous work (*12*). [1-^{14}C]IPP was purchased from Amersham. (2*E*)-3-Methyl-2-alkenyl diphosphate, (2*E*)-3-methyl-2,6-heptadienyl diphosphate, and (all-*E*)-3,7-dimethyl-2,4,6-octatrienyl diphosphate were the same preparations as used in the previous work (*17, 18*). The plasmids pI-9, pI-11, and pI-23, which express mutated GGPP synthases, I-9, I-11, and I-23, in *Escherichia coli*, respectively, and pHK119 were described in our previous paper (*16*). All other chemicals were of analytical grade.

Construction of Mutated GGPP Synthases by Site-Directed Mutagenesis—Site-directed mutagenesis was performed by the Kunkel method (*19*). A single-strand wild-type GGPP synthase gene, used as a template in the mutagenesis reaction, was prepared by M13KO7 helper phage infection of XL1-Blue MRF' cells (Stratagene) that contained pHK119 (*16*), which contained the *S. acidocaldarius* GGPP synthase gene. The resulting anti-sense single-strand DNA template was isolated, purified by standard methods, and sequenced to confirm its identity. The synthetic sense oligonucleotides designed to produce the desired point mutations were as follows: OL-1, 5′-ACGTGAGATCGGGTCAAGGTAT-3′; OL-2, 5′-CTTACGTGAGCGCGGGTCAAGGTAT-3′; OL-3, 5′-TGTCTTACGTACGATCGGGTCAAGGTAT-3′. After mutagenesis, all mutants were confirmed by DNA sequencing.

Preparation of Mutated GGPP Synthase—*E. coli* DH5α was transformed with each of the plasmids coding GGPP synthase, and cultured according to the methods described previously (*20*). The cells were harvested and disrupted by sonication in 50 mM Tris-HCl buffer, pH 7.0, containing 10 mM 2-mercaptoethanol and 1 mM EDTA. The homogenate was heated at 55°C for 120 min and then centrifuged at 100,000 × *g* for 10 min. The supernatant, which contained no prenyltransferase activity derived from the host, was used as a crude enzyme. Expression levels and purification levels of all mutated FPP synthases were confirmed by SDS-PAGE (12.5%) with Coomassie Brilliant Blue staining (data not shown).

Measurement of Prenyltransferase Activity—The assay mixture contained, in a final volume of 1 ml, 1.25 nmol of [1-^{14}C]IPP (740 GBq/mol), 1.25 nmol of the indicated allylic substrate [DMAPP, GPP, (all-*E*)-FPP, artificial substrates], 5 μmol of $MgCl_2$, 10 μmol of phosphate buffer (pH 5.8), and a suitable amount of enzyme. This mixture was incubated at 55°C for the indicated time, and the reaction was stopped by chilling the reaction mixture in an ice bath. The mixture was shaken with 3.5 ml of 1-butanol which had been saturated with H_2O. The butanol layer was washed with water saturated with NaCl, and radioactivity in the butanol layer was measured.

Product Analysis—After enzymatic reaction at 55°C, the polyprenyl diphosphates were extracted with 1-butanol, and then the solvent was evaporated off under an N_2 stream. The resulting polyprenyl diphosphates were treated with acid phosphatase according to the method of Fujii *et al.* (*21*). The hydrolysates were extracted with pentane and analyzed by reversed-phase thin-layer chromatography using a precoated TLC plate, LKC-18, developed with acetone/H_2O (9 : 1). Authentic standard alcohols were visualized with iodine vapor, and the distribution of radioactivity was detected by a Bio-image analyzer BAS2000 (Fuji Film).

RESULTS AND DISCUSSION

We previously prepared dozens of mutated *S. acidocaldarius* GGPP synthases that have mutations in the region from position 170 to 173 (*16*), which is thought to be a part of a putative substrate binding region of prenyltransferase (*22*). We analyzed the mutated GGPP synthases with respect to substrate specificity and intermediate accumulation. Two of the mutated GGPP synthases, I-9 and I-11, which have substitutions of four amino acids in the region (Fig. 2), showed considerably lower activities toward primer substrates with a short prenyl chain, DMAPP and GPP, than the wild-type enzyme (Fig. 3, Ref. *9*). In particular, I-11 scarcely accepts DMAPP as an allylic substrate. In order to examine the specificity for allylic substrates precisely, we employed a series of artificial allylic substrates, 3-methyl-2-alkenyl diphosphates, **1**–**9** (Fig. 4), each of which has an *E*-alkyl group with a different chain length. These artificial substrates were previously used for analyses of the substrate specificity of eubacterial and plant GGPP synthases (*1, 23*). After the enzyme reaction with each of these allylic substrates and [1-^{14}C]-IPP, the radioactivity of butanol-extractable materials was evaluated (Fig. 5A). The wild-type enzyme accepted all 3-methyl-2-alkenyl diphosphates tested as allylic primer substrates to form butanol-extractable materials, although the activities for those with short alkyl chains, **2**–**4**, were relatively low. Although this profile of substrate specificity is similar to those of GGPP synthases from *M. luteus* and pumpkin (*1*), GGPP synthases from *M. luteus* and pumpkin show high activities toward 1 (DMAPP) and **6**. In the case of archaeal enzyme from *S. acidocaldarius*, **8** was the most active among the artificial substrates. Figure 5B shows an

analysis of products formed by the wild-type enzyme. The product obtained from the C_6-substrate **2** gave two radioactivity spots. The major one migrated slightly more slowly than (all-*E*)-geranylgeraniol (GGOH) and the other migrated more slowly than (all-*E*)-farnesol (FOH), indicating that the products were C_{21}- and C_{16}-compounds resulting from condensations with three and two molecules of IPP, respectively. In the reactions using **3** or **4** as the allylic primer, products attributable to condensations of these substrates with three molecules of IPP were not produced. When the C_9 substrate **5** was used, the main product was the C_{19}-compound, and production of the C_{14}-compound was also observed. The longer the alkyl chain of the substrates became, the more product formation by single condensation was increased. The product formed by condensations involving two molecules of IPP was decreased. Reactions with **8** and with **9** gave C_{17}- and C_{18}-compounds, respectively, which were formed by the single condensation of IPP. In all cases, the carbon numbers of products were between C_{21} and C_{14}, and the enzyme never produced a condensation product having a carbon number beyond C_{21}.

Recently we have elucidated the mechanism which determines the chain-length of the ultimate product of prenyltransferase (*14, 15, 24, 25*). In the case of *S. acidocaldarius* GGPP synthase, the side chain of the phenylalanine residue at position 77 directly contacts the product and prevents further condensation (*15*). This mechanism fits in with the data described above, indicating that the wild-type GGPP synthase from the archaea recognizes the length of the hydrocarbon chain to determine the final product. The artificial substrates employed lack one or two double bonds in the natural substrates, GPP or FPP, thus the hydrocarbon chains of the products are more flexible than that of GGPP. This enables the enzyme to generate products with longer hydrocarbon chains than that of GGPP, *e.g.* the C_{21}-product derived from the reaction with **7**. However, the hydrocarbon chains of the products are not as long as that of geranylfarnesyl diphosphate, a polyprenyl diphosphate with five isoprene units.

The mutated GGPP synthases, I-9 and I-11, show lower activities for the substrates with short chain lengths than the wild-type enzyme. In particular, the mutated enzyme I-11 could not accept substrates **1**, **2**, **3**, and **4** (Fig. 5, A and C). These data clearly show that the mutated region of the GGPP synthase is involved in the recognition of allylic substrates with short alkyl chains. The product derived from the C_9-substrate **5** was the C_{19}-compound, which was attributable to the condensation of the substrate with two molecules of IPP. In the reaction with **6**, two products, C_{20} and C_{15}, were obtained. As the alkyl chain became longer, the product distribution was also changed in a manner similar to that observed with the wild-type enzyme. As shown in Fig. 5C, the chain lengths of the final products obtained from I-11 were the same as in the case of the wild-type enzyme. In our previous study we isolated

	170	171	172	173
Wild type	- Asp -	Met -	Ile -	Ser -
I-9	- **Glu** -	**Pro** -	**Ala** -	**Met** -
I-11	- **Glu** -	**Pro** -	**Arg** -	**Val** -
I-23	- **Glu** -	**Pro** -	**Ala** -	**Arg** -
OL-1	- Asp -	**Pro** -	Ile -	Ser -
OL-2	- Asp -	**Pro** -	**Arg** -	Ser -
OL-3	- Asp -	**Pro** -	Ile -	**Val** -

Fig. 2. **Amino acid sequences of mutated GGPP synthases.** Amino acid sequences from 170 to 173 of the wild-type and six mutated GGPP synthases are shown. Mutated amino acids are shown in boldface.

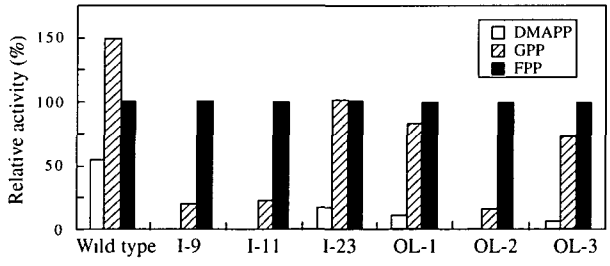
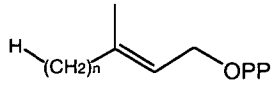
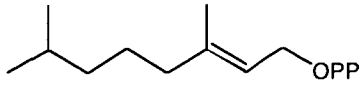


Fig. 3. **Natural substrate specificity of mutated GGPP synthases.** The reaction with 1.25 μM [1-^{14}C]IPP (0.74 GBq/mmol), 1.25 μM allylic substrate, and a suitable amount of enzyme was carried out, and the radioactivity of 1-butanol-extractable materials was determined as described in "EXPERIMENTAL PROCEDURES." Activities relative to that for FPP are indicated in the figure.

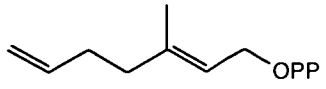


1～**9** : (2*E*)-3-methyl-2-alkenyl diphosphate

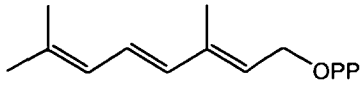
1 : n=1 (DMAPP)
2 : n=2
3 : n=3
4 : n=4
5 : n=5
6 : n=6
7 : n=7
8 : n=8
9 : n=9



10 : (2*E*)-3,7-dimethyl-2-octenyl diphosphate



11 : (2*E*)-3-methyl-2,6-heptadienyl diphosphate



12 : (all-*E*)-3,7-dimethyl-2,4,6-octatrienyl diphosphate

Fig. 4. **Artificial allylic substrates.**

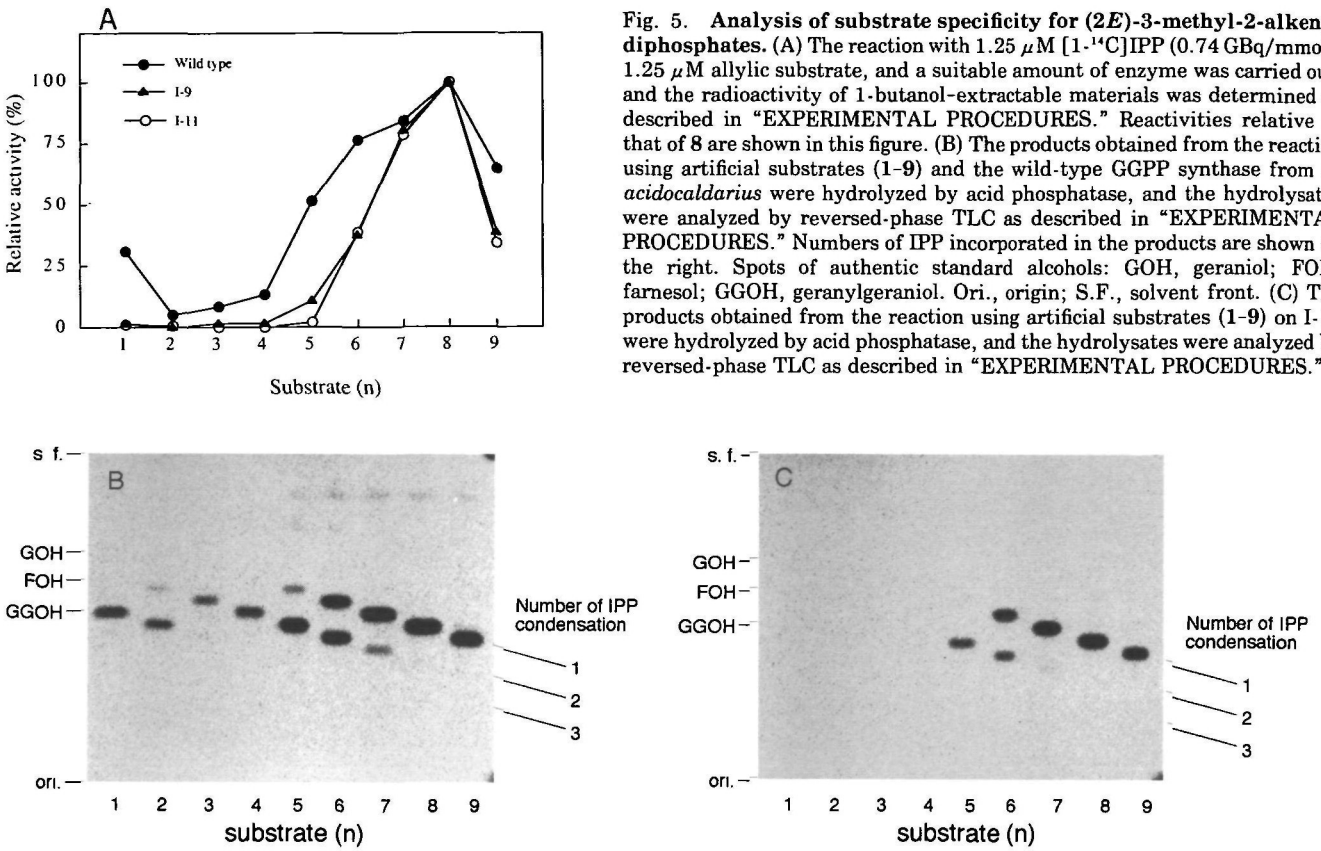


Fig. 5. **Analysis of substrate specificity for (2*E*)-3-methyl-2-alkenyl diphosphates.** (A) The reaction with 1.25 μM [1-^{14}C]IPP (0.74 GBq/mmol), 1.25 μM allylic substrate, and a suitable amount of enzyme was carried out, and the radioactivity of 1-butanol-extractable materials was determined as described in "EXPERIMENTAL PROCEDURES." Reactivities relative to that of **8** are shown in this figure. (B) The products obtained from the reaction using artificial substrates (**1**–**9**) and the wild-type GGPP synthase from *S. acidocaldarius* were hydrolyzed by acid phosphatase, and the hydrolysates were analyzed by reversed-phase TLC as described in "EXPERIMENTAL PROCEDURES." Numbers of IPP incorporated in the products are shown on the right. Spots of authentic standard alcohols: GOH, geraniol; FOH, farnesol; GGOH, geranylgeraniol. Ori., origin; S.F., solvent front. (C) The products obtained from the reaction using artificial substrates (**1**–**9**) on I-11 were hydrolyzed by acid phosphatase, and the hydrolysates were analyzed by reversed-phase TLC as described in "EXPERIMENTAL PROCEDURES."

TABLE I. **Activity of the wild-type GGPP synthase and mutant I-11 for several allylic substrates.** The reaction with 1.25 μM [1-^{14}C]IPP (0.74 GBq/mmol), 1.25 μM allylic substrate, and a suitable amount of enzyme was carried out, and the radioactivity of 1-butanol-extractable materials was determined as described in "EXPERIMENTAL PROCEDURES." Activities relative to that for GPP are indicated.

Enzyme	Allylic substrate						
	GPP	**10**	**5**	**6**	**11**	**4**	**12**
Wild type	100[a]	62.3	63.7	94.9	24.2	21.5	nd[b]
I-11	100[a]	15.5	12.6	25.3	nd[b]	nd[b]	nd[b]

[a]Relative activity. [b]Not detected.

several mutated *S. acidocaldarius* GGPP synthases having replacements outside the region from positions 170 to 173, which produce final products longer than GGPP (*15*). Thus, the region from positions 170 to 173 is probably not responsible for determination of the chain length of the final product. Moreover, in I-11, the amount of C_{14}-compound formed from **5** and that of C_{21}-compound from **7** were lower than those in the case of the wild-type enzyme. These results might indicate that the mutations also cause sharpening of the product distribution.

Both mutated GGPP synthases examined in this study contain four amino acid alterations ranging from positions 170 to 173. Because the two mutants have a common substitution of a proline residue at position 171, their properties seem to be attributable to the proline residue. A mutated GGPP synthase I-23, which was isolated in our previous work and has the same proline substitution at position 171 (*16*), also shows reduced activities for primers with short chains (Figs. 2 and 3). In addition, all other mutated GGPP synthases that did not have a proline residue at position 171 could accept DMAPP as a substrate to give GGPP (*16*). Therefore, substitution of proline at position 171 seems to be essential for the change of substrate specificity. In order to confirm the effective substitutions for substrate recognition, three mutated GGPP synthases that have mutations within positions 172 to 173 in addition to M171P mutation were prepared, and their properties were analyzed (Figs. 2 and 3). Replacement of aspartate at position 170 with glutamate is not important for the change because OL-1, 2, and 3 showed similar properties to I-9, I-11, and I-23. Replacement of isoleucine with arginine at position 172 is effective for the change in substrate specificity because the activity for DMAPP of OL-2 is less than that of OL-1. On the other hand, replacement of serine with valine at position 173 seems not to be important because the substrate recognition of OL-1 is similar to that of OL-3. However, the difference in substrate specificity between I-9 and I-23 seems to be attributable to the residues Met and Arg at position 173. These results indicate that substitution of proline at position 171 mainly causes the change in the allylic substrate specificity and that mutations at positions 172 and 173 also play a part.

We also analyzed the substrate specificity of I-11 and the wild-type enzyme using other artificial allylic diphosphates (Fig. 4 and Table I). (2*E*)-3,7-Dimethyl-2-octenyl diphosphate, **10** was accepted by both enzymes as an allylic substrate, and two products, 3,7,11-trimethyl-2,6-undecadienyl diphosphate and 3,7,11,16-tetramethyl-2,6,10-hex-

adecatrienyl diphosphate, which are formed by the condensations of one and two molecules of IPP, respectively, were obtained (data not shown). The susceptibility as a substrate of **10** is similar not to that of the C_{10}-substrate **6**, but to those of the C_9-substrate **5** in both enzymes. These data indicate that GGPP synthase of *S. acidocaldarius* recognizes the length of the alkyl chain rather than its carbon number. Moreover, the reactivity of **10** is lower than that of GPP in both enzymes, indicating that the double bond at position 6 is involved in substrate recognition. The wild-type enzyme accepted (2*E*)-3-methyl-2,6-heptadienyl diphosphate, **11**, with similar reactivity to that for the C_8-substrate **4** to give 3,7,11-trimethyl-2,6,10,14-heptadecatetraenyl diphosphate. These data show that the double bond at the ω-end in the allylic substrate is not important for the recognition. Moreover, as expected from the result that I-11 does not accept the C_8-substrate **4** as a substrate, I-11 could not catalyze the condensation of **11**. No enzyme could accept (all-*E*)-3,7-dimethyl-2,4,6-octatrienyl diphosphate, **12**.

In summary, *S. acidocaldarius* GGPP synthase determines the final product by recognizing the length of its hydrocarbon chain. On the other hand, the result that mutated enzyme I-11 does not accept short allylic substrates but forms products with the same length as those of the wild-type enzyme indicates that GGPP synthase also recognizes the chain length of the substrate at a region different from that for product recognition.

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