

The Carboxyl-terminal Region of the Geranylgeranyl Diphosphate Synthase is Indispensable for the Stabilization of the Region Involved in Substrate Binding and Catalysis

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Rat geranylgeranyl diphosphate synthase (GGPS) and its deletion mutants from the carboxyl terminus were analysed using *Escherichia coli* harbouring pACYC-crtIB, which contains *crtI* and *crtB* encoding the carotenoid biosynthetic enzymes. Mutants (Δ -4, -8, -12 and -16) produced lycopene-derived red colour, but mutants (Δ -17, -18, -19, -20, -23, -57 and -70) did not. The histidine-tagged mutants (Δ -4, -8, -12 and -16) were overexpressed in *E. coli* BL21 (DE3) and purified in a stable form by nickel affinity chromatography except for one mutant (Δ -16). The farnesyl-transferring activities of wild-type GGPS, Δ -4, -8 and -12 mutants were relatively in a ratio of 1.0, 0.84, 0.26 and 0.0015. Each K_m value of the four recombinants were estimated to be 0.71, 2.0, 2.8 and 55 μ M for farnesyl diphosphate and to be 2.9, 5.1, 56 and >100 μ M for isopentenyl diphosphate, respectively. Allylic substrate specificities of these recombinants were estimated by quantitative analysis of the products, revealing that Δ -8 and -12 mutants lack the ability to accept dimethylallyl and geranyl diphosphates compared to wild-type GGPS and Δ -4 mutant. These results suggest that the KMFTEENE residing on the carboxyl-terminal sequence of GGPS stabilizes the active region involved in the substrate binding and catalysis.

Key words: carotenoid, deletion mutants, geranylgeranyl diphosphate synthase, prenyltransferase, structure and activity.

Abbreviations: DMAPP, dimethylallyl diphosphate; FPP, farnesyl diphosphate; GPP, geranyl diphosphate; GGPP, geranylgeranyl diphosphate; GGPS, geranylgeranyl diphosphate synthase; IPP, isopentenyl diphosphate.

Geranylgeranyl diphosphate synthase (GGPS) in mevalonate metabolism catalyses the synthesis of geranylgeranyl diphosphate (GGPP) from farnesyl diphosphate (FPP) and isopentenyl diphosphate (IPP) (1). The product, GGPP, is utilized as a prenyl precursor for the synthesis of geranylgeranylated proteins such as Rho, Rac and Rab (2–4). Mammalian GGPSs have been cloned from human (5) and mouse (6), and found to be a protein of 300 amino acids. Recently, we have found six rat GGPS mRNAs and analysed their sequences together with its genome sequence (7), indicating the presence of alternative splice of GGPS mRNA. Six mRNAs are classified into three encoding the 300, 275 and 267 amino acids, which are identical in the amino (N)-terminal 230 amino acid sequences but different in the following carboxyl(C)-terminal sequences. In expression experiments with *E. coli* or HeLa cells, one protein (300 amino acids) was active, but the others (275 and 267 amino acids) were inactive. This led us to speculate that the C-terminal residues of GGPS (300 amino acids) might be important for the active structure of GGPS,

because the two shorter GGPS (275 and 267 amino acids) containing five conserved motifs (I, II, III, IV and V) common to all-*trans*-prenyltransferase family (8) were both inactive.

So far, 3D-structure analysis of farnesyl diphosphate synthase (FPS) and octaprenyl diphosphate synthase (OPS) has been reported (9, 10), and their C-terminal regions are located far from a core pocket constituting the active sites. It might be possible to assume the structure of GGPS in analogy to those of FPS and OPS, because not only GGPS but also FPS and OPS belong to all-*trans*-prenyltransferase family. However, these C-terminal amino acid sequences of FPS, OPS and GGPS are completely different from one another, and the role of C-terminal regions remains unclear. In this article, we prepared several deletion mutants of rat GGPS (300 amino acids) focusing on the C-terminal region and characterized them. We report that the C-terminal region of GGPS is indispensable for the stabilization of its active structure.

MATERIALS AND METHODS

[1-¹⁴C]IPP (58 μ Ci/ μ mol) was purchased from Amersham Pharmacia Biotech. Dimethylallyl diphosphate (DMAPP), geranyl diphosphate (GPP) and FPP

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were prepared as described in a previous report (11). pACYC-crtIB was a kind gift from Dr Hemmi, Tohoku University. KOD-plus DNA polymerase was obtained from TOYOBO. PCR primers were synthesized at KURABO. All other chemicals were of reagent grade. Silica gel 60 thin-layer plates (Merck #11845) were obtained from Merck.

Expression of Rat His-tagged GGPS Recombinant in *E. coli* Cells—The pCR2.1 containing full-length rat GGPS cDNA was used as a template for PCR (7). PCR with a combination of an *Nde*I-sense and a *Bam*HI-antisense primers were done under the following conditions: 2 s at 94°C, 30 s at 57°C and 1 min at 68°C for 30 cycles. The PCR products corresponding to rat GGPS and its deletion mutants at the C-terminal region were ligated into *Nde*I and *Bam*HI sites of pET-15b (Novagen) to generate pET-rGGPS and pET-rGGPS-mutant. The plasmid sequences were confirmed, and *E. coli* BL21 (DE3) cells transformed with each pET plasmid were grown to an A_{600} of 0.6 in M9YG medium at 30°C. Isopropyl 1-thio- β -D-galactopyranoside (IPTG) was added to the final concentration of 0.3 mM, and cells were cultured at 18°C for 6 h. Cells were collected by centrifugation at 10,000g for 10 min and stored under -80°C before use. Whole cell extracts were separated by SDS-polyacrylamide gel electrophoresis and stained with Coomassie brilliant blue. Histidine(His)-tagged proteins were purified by nickel affinity column chromatography as described in the previous report (7).

Enzyme Assay and Product Analysis—The standard assay mixture contained, in a final volume of 25 μ l, 50 mM potassium phosphate buffer (pH 7.0), 2 mM dithiothreitol, 5 mM $MgCl_2$, 20 μ M [$1-^{14}C$]IPP, 20 μ M DMAPP, GPP or FPP, 0.1% (w/v) bovine serum albumin and an appropriate amount of enzyme fraction. The mixture was incubated at 37°C for 15 min and terminated by the addition of 75 μ l of concentrated HCl/methanol (1:4) followed by a 15 min incubation at 37°C. The hexane-soluble hydrolysates were analysed in a liquid scintillation fluid. For product analysis, the aliquot of the reaction mixture was directly applied on a silica-gel thin-layer plate and developed in a solvent system of isopropanol/ammonia/water (6:3:1). The positions of authentic standards were visualized with iodine vapour. The radioactivity of products developed on thin-layer chromatography was determined with a Fuji Bioimage analyser BAS 1000.

Carotenoid-colour Production Test—For *in vivo* GGPS assay, *E. coli* JM109 (DE3) cells harbouring pACYC-crtIB (12), which contains *crtI* and *crtB* encoding carotenoid biosynthetic enzymes, were transformed with each pET plasmid and plated on LB plates containing 100 μ g/ml ampicillin and 12 μ g/ml tetracycline. Resistant colonies were replated on LB plates containing the same antibiotics and cultured at 20.5°C for 4 days, and the change in colour of the colonies due to the accumulation of lycopene was examined.

RESULTS AND DISCUSSION

Figure 1 shows the alignment of amino acid sequences for the human and mouse GGPSs together with rat GGPS. These mammalian GGPSs (300 amino acids) contain five conserved motifs (I, II, III, IV and V), which are common to all-*trans*-prenyltransferase family, in the N-terminal sequence (position 1–230). To understand whether the C-terminal region (231–300) as well as the N-terminal region (1–230) is important for the mammalian GGPS activity, we constructed expression vectors (pET15b) encoding wild-type GGPS (Δ -0) and its deletion mutants from the C-terminus (Δ -4, -8, -12, -16, -17, -18, -19, -20, -23, -57 and -70), and tried to express each using carotenoid-colour producing system (Fig. 2). Lycopene-derived red colour was observed in colonies expressing Δ -0, -4, -8, -12 and -16, but not in those expressing Δ -17, -18, -19, -20, -23, -57 and -70. These results indicate that the region of 17 amino acid residues (LVALVKHLSKMFTEENE) from the C-terminus is important for keeping GGPS at least in an active form.

Further, we tried to characterize Δ -0, -4, -8, -12 and -16. His-tagged recombinants were overexpressed by IPTG in *E. coli* BL21 (DE3) and the cells were disrupted by sonication followed by centrifugation. Δ -0, -4, -8 and -12 were recovered in soluble fractions, but the Δ -16 was recovered in precipitate fractions. The insoluble Δ -16 was active as assayed with the combination of [$1-^{14}C$]IPP and FPP, but the activity was extremely low compared to other mutants. The soluble Δ -0, -4, -8 and -12 were further purified by the nickel affinity chromatography and assayed with the same substrate combination. The His-tagged and thrombin-cleaved forms of the enzyme were tested for activity and it was found that the presence of the tag had no significant effect on the enzyme activity (data not shown). Table 1 shows the

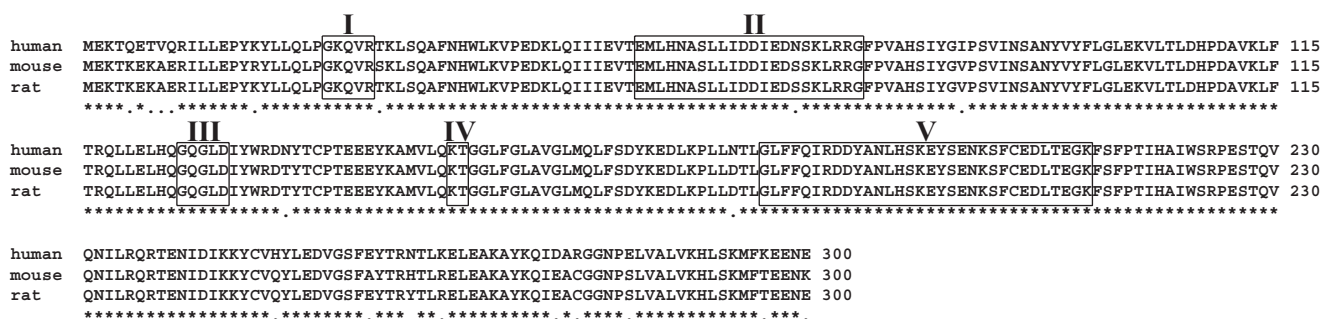


Fig. 1. Amino acid sequences alignment of GGPS. Amino acid sequences for human, mouse and rat GGPSs are shown. Five regions (I, II, III, IV and V) in each GGPS show five conserved motifs common to all-*trans* prenyltransferases.

C-terminal sequences of rat recombinant GGPS used in the present study

MEKT-(I)-(II)-(III)-(IV)-(V)-TQVQNILRQRTENIDIKKYCVQYLEDVGSFEYTRYTLRELEAKAYKQIEACGGNPSLVALVKHLSKMFT	Δ-0
MEKT-(I)-(II)-(III)-(IV)-(V)-TQVQNILRQRTENIDIKKYCVQYLEDVGSFEYTRYTLRELEAKAYKQIEACGGNPSLVALVKHLS	Δ-4
MEKT-(I)-(II)-(III)-(IV)-(V)-TQVQNILRQRTENIDIKKYCVQYLEDVGSFEYTRYTLRELEAKAYKQIEACGGNPSLVALVKHLS	Δ-8
MEKT-(I)-(II)-(III)-(IV)-(V)-TQVQNILRQRTENIDIKKYCVQYLEDVGSFEYTRYTLRELEAKAYKQIEACGGNPSLVALV	Δ-12
MEKT-(I)-(II)-(III)-(IV)-(V)-TQVQNILRQRTENIDIKKYCVQYLEDVGSFEYTRYTLRELEAKAYKQIEACGGNPSL	Δ-16
MEKT-(I)-(II)-(III)-(IV)-(V)-TQVQNILRQRTENIDIKKYCVQYLEDVGSFEYTRYTLRELEAKAYKQIEACGGNPS	Δ-17
MEKT-(I)-(II)-(III)-(IV)-(V)-TQVQNILRQRTENIDIKKYCVQYLEDVGSFEYTRYTLRELEAKAYKQIEACGGNP	Δ-18
MEKT-(I)-(II)-(III)-(IV)-(V)-TQVQNILRQRTENIDIKKYCVQYLEDVGSFEYTRYTLRELEAKAYKQIEACGGN	Δ-19
MEKT-(I)-(II)-(III)-(IV)-(V)-TQVQNILRQRTENIDIKKYCVQYLEDVGSFEYTRYTLRELEAKAYKQIEACGG	Δ-20
MEKT-(I)-(II)-(III)-(IV)-(V)-TQVQNILRQRTENIDIKKYCVQYLEDVGSFEYTRYTLRELEAKAYKQIEA	Δ-23
MEKT-(I)-(II)-(III)-(IV)-(V)-TQVQNILRQRTENIDI	Δ-57
MEKT-(I)-(II)-(III)-(IV)-(V)-TQV	Δ-70

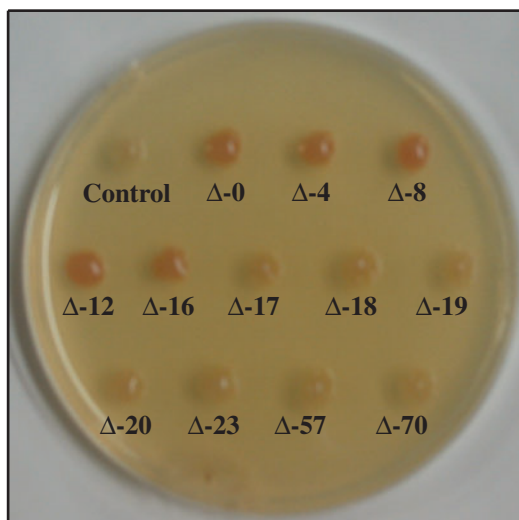


Fig. 2. **Upper: Amino acid sequences of GGPS and its mutant.** Δ-0 corresponds to the rat GGPS as shown in Fig. 1. Each mutant protein contains a His-tagged 20 amino acid sequence at the amino-terminus. **Lower: Carotenoid-colour production test using GGPS and its mutants.** *Escherichia coli*

JM109 (DE3) cells harbouring pACYA-crtIB and the pET plasmid expressing His-tagged GGPS and its mutants were replated on an LB plate containing appropriate antibiotics and cultured at 20.5°C for 4 days.

Table 1. **Kinetic parameters.**

Enzyme	Specific activity		K_m (FPP) μM	K_m (IPP) μM
	nmol/min/mg	fold		
Δ-0	117	(1.0)	0.71	2.9
Δ-4	98.3	(0.84)	2.0	5.1
Δ-8	30.6	(0.26)	2.8	56
Δ-12	0.176	(0.0015)	55	>100

For the estimation of specific activity, the assay was done with a combination of 20 μM [1-¹⁴C]IPP and 20 μM FPP. For the estimation of K_m values, the constant concentration of 20 μM [1-¹⁴C]IPP or 20 μM FPP was used in the case of Δ-0 and Δ-4, and the constant concentration of 100 μM [1-¹⁴C]IPP or 100 μM FPP was used in the case of Δ-8 and Δ-12.

specific activities and K_m values of Δ-0, -4, -8 and -12. The specific activities were relatively estimated to be 1.0, 0.84, 0.26 and 0.0015 in the order. The K_m values for FPP were estimated to be 0.71, 2.0, 2.8 and 55 μM and those for IPP were also estimated to be 2.9, 5.1, 56 and >100 μM in the order. Deletion of the four amino acid residues (EENE) caused about 2-fold decrease in the affinity for both substrates, but the catalytic activity (Δ-4) was almost similar to that of wild-type GGPS.

Deletion of the following four amino acid residues (KMFT) caused a significant decrease in the affinity for IPP. Further deletion of the following four amino acid residues (KHLS) caused a significant decrease in the catalytic activity as well as in the affinity for both substrates. These results suggest that the substrate binding and catalytic sites responsible for the reaction between FPP and IPP are stabilized with the C-terminal regions.

Using Δ-0, -4, -8 and -12, we further analysed enzymatic products from 20 μM allylic substrate (DMAPP, GPP or FPP) and 20 μM [1-¹⁴C]IPP. Assay mixture containing each recombinant (40 ng) were incubated for 15 min, applied to silica gel thin-layer plates and developed in a solvent system of isopropanol/ammonia/water (6:3:1). As shown in Fig. 3A and B, Δ-4 was similar to Δ-0 with respect to substrate specificity and product distribution. In the case of Δ-8, and Δ-12, the radioactive products were too small to detect (data not shown). Concerning the Δ-8, we further analysed products in the reaction using 200 ng instead of 40 ng. After incubation for 30 min, GGPP and FPP were detected as products in the geranyl-transferring reaction (Fig. 3C lane 3). Concerning the Δ-12, we analysed products in the reaction using 1,000 ng instead of 40 ng.

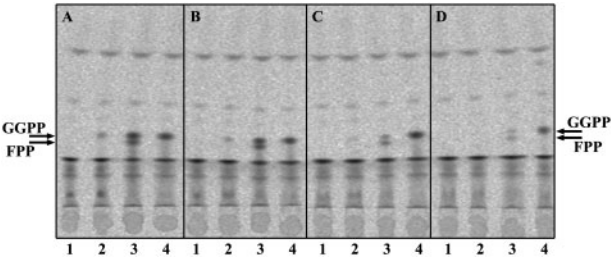


Fig. 3. Silica-gel thin-layer chromatograms of enzymatic products. The supernatant fraction obtained by the centrifugation of the crude cell extracts containing His-tagged GGPS (Δ -0) and its deletion mutants (Δ -4, Δ -8 and Δ -12) were purified by the nickel affinity chromatography and assayed with a combination of allylic substrate and [$1\text{-}^{14}\text{C}$]IPP. Lanes 1, 2, 3 and 4 indicate the reaction with IPP, IPP+DMAPP, IPP+GPP and IPP+FPP, respectively. For the development, a solvent system of isopropanol/ammonia/water (6:3:1) was used. A (Δ -0), B (Δ -4), C (Δ -8), D (Δ -12).

After incubation for 180 min, GGPP was detected as products in farnesyl-transferring reaction (Fig. 3D lane 4), and GGPP and FPP were also detected as products in geranyl-transferring reaction (Fig. 3D lane 3). Table 2 summarizes the quantitative analysis of product distribution depending on allylic substrates. GGPP-synthesizing activities from DMAPP, GPP and FPP with IPP were estimated to be in the ratio of 0.02:0.61:1.0 and 0.02:0.45:1.0 for Δ -0 and -4, respectively (Table 2). As for Δ -8 and Δ -12, GGPP-synthesizing activities were estimated to be in the ratio of 0:0.02:1. and 0:0.03:1, respectively (Table 2). It might be possible to explain that the remarkably decreased GGPP-synthesizing activity from GPP of Δ -8 compared with that of Δ -4 is due to the decrease in the affinity for IPP. These results suggest that the stabilization with the C-terminal regions is accompanied by enabling to accept DMAPP and GPP as substrate.

The present study shows that the activities of rat GGPS (300 amino acids) are stabilized by its C-terminal amino acid region. Deletion of 17 amino acid residues (284–300, LVALVKHLSKMFTEENE) causes the loss of GGPS activity, suggesting that the N-terminal 284 amino acids region containing five conserved motifs is requisite for constituting its active structure and that the following 16 amino acid residues (285–300, VALVKHLSKMFTEENE) are responsible for the construction of fully active GGPS. As shown in Fig. 1, the C-terminal regions of human and mouse GGPSs are almost the same as that of the rat enzyme in the sequence and length. Therefore, the importance of the C-terminal region shown in the rat GGPS might be similar to the case of human and mouse GGPSs. One amphibian GGPS (*Xenopus tropicalis*; GenBank, CR761149) has been predicted to be a protein of 296 amino acids, which is almost the same as mammalian enzymes (300 amino acids) in the amino acid sequence. The predicted C-terminal 13 amino acid residues (284–296, LASLIEQLSKIYQ) are similar to that of rat Δ -4 (284–296, LVALVKHLSKMFTE). Since Δ -4 shows a weaker affinity for IPP or FPP than Δ -0, it is very interesting to examine whether the amphibian GGPS

Table 2. Distribution of enzymatic products.

Enzyme	Allylic primer	Enzymatic products					
		GPP	FPP		GGPP		
Δ -0	+DMAPP	0	27	(0.00)	379	(0.02)	
	+GPP	0	3,450	(0.68)	6,192	(0.61)	
	+FPP	0	0		5,075	(1.00)	
Δ -4	+DMAPP	0	66	(0.00)	240	(0.02)	
	+GPP	0	2,921	(0.70)	3,762	(0.45)	
	+FPP	0	0		4,200	(1.00)	
Δ -8	+DMAPP	0	2	(0.00)	15	(0.00)	
	+GPP	0	722	(0.10)	336	(0.02)	
	+FPP	0	0		6,927	(1.00)	
Δ -12	+DMAPP	0	0	(0.00)	0	(0.00)	
	+GPP	0	197	(0.10)	115	(0.03)	
	+FPP	0	0		1,972	(1.00)	

The radioactive products as shown in Fig. 3A (Δ -0), 3B (Δ -4), 3C (Δ -8) and 3D (Δ -12) were quantitatively analysed. The figures in parentheses indicate the reaction velocities for the $\text{C}_5 \rightarrow \text{C}_{20}$, $\text{C}_{10} \rightarrow \text{C}_{20}$ and $\text{C}_{15} \rightarrow \text{C}_{20}$ steps of the reaction calculated on the assumption that the specific activities of GGPP formed from [$1\text{-}^{14}\text{C}$]IPP (C_5) with DMAPP (C_5) and with GPP (C_{10}) relative to that of GGPP formed from [$1\text{-}^{14}\text{C}$]IPP with FPP (C_{15}) are 3 and 2, respectively.

also shows a weaker affinity for IPP or FPP than mammalian GGPS to understand the functional evolution of GGPS.

It should be noted that the C-terminal amino acid residues of GGPS, which are not in the centre of activation, are concerned with the enzyme activity. In case of plant H^+ -ATPase (13), it has been reported that the C-terminal deletion affects the affinity for ATP. In case of the C-terminal deletion mutants of *Bacillus stearothermophilus* FPS, a similar decline of the enzyme activity has been observed (Koyama, T., personal communication). The C-terminal regions in these enzymes are also requisite to maintain them in stable and active forms. As the presence study shows, it is clear that the length of the C-terminal amino acid residues is important for the stable form of active GGPS. However, whether the sequence of the C-terminal amino acid residues is also important is unclear.

More recently, Kavanagh *et al.* (14) reported the crystal structure of human GGPS. The crystal structure was analysed to be a hexamer consisting of three dimers. On the other hand, Kuzuguchi *et al.* (5) reported that the human GGPS in solution is an octamer. Presumably, the dimer forms would be assembled to hexamer in crystal or to octamer in solution. The molecular structures of rat GGPS might be also similar to those of human GGPS, since primary amino acid sequences are almost similar between them as shown in Fig. 1. The C-terminal region of human GGPS similar to those of rat GGPS is found to be located to neither inter-monomer nor inter-dimer regions of human GGPS hexamer in crystal. Since it is likely that GGPS is an octamer in solution, how the C-terminal regions stabilize substrate binding and catalytic sites of octamers in solution is still unknown. The study of chromatographic behaviours on the gel filtration of rat GGPS and several mutants shown in the present study will help to learn more about the structure and activity relationship of GGPS.

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