Purification of geranylgeranyl diphosphate synthase from bovine brain

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Geranylgeranyl diphosphate (GGPP) synthase was purified to homogeneity from bovine brain in a one-step affinity column procedure. For the construction of the affinity column, a farnesyl diphosphate (FPP) analog, O-(6-amino-1-hexyl)-P-farnesylmethyl phosphonophosphate, was synthesized and linked to the spacer of the matrix of Affigel 10 via the amino group. The native enzyme appeared to be homooligomer (150–195 kDa) with a molecular mass of the monomer of 37.5 kDa. The pI for the enzyme was 6.2. The Km values for dimethylallyl diphosphate (DMAPP), geranyl diphosphate (GPP) and FPP were estimated to be 33 μM, 0.80 μM and 0.74 μM, respectively. The Km value for isopentenyl diphosphate (IPP) in the presence of both IPP and FPP mixture was 2 μM. The ratio of the reaction velocity for formation of GGPP from DMAPP, GPP or FPP was 0.004:0.145:1. The intermediate IPP was formed in the reaction with GPP as an allyclic primer. FPP synthase catalyzing the formation of FPP from DMAPP and IPP was also purified to homogeneity from the same organ by a similar affinity chromatography procedure using a GPP analog, O-(6-amino-1-hexyl)-P-geranyl methyl phosphonophosphate as a ligand. The enzyme was a homodimer with a monomeric molecular mass of 40.0 kDa. These results indicate that GGPP, a lipid precursor for the biosynthesis of a majority of prenylated proteins, is synthesized from DMAPP and IPP by the action of FPP synthase catalyzing the reactions C5→C15 followed by the action of GGPP synthase catalyzing the reaction C15→C20.

A number of prenylated proteins have been found in various organisms [1–5]. These include Ras proteins, Ras-related small G-proteins, nuclear lamin proteins, γ-subunit of heterotrimeric large G-proteins, and fungal mating pheromones. The prenylation of these proteins has been reported to be essential for their function in the cells [6–8] and, by identifying protein prenyltransferases in yeast and mammalian brains [9], the direct precursors of prenyl moiety have been established to be FPP and GGPP. Three types of protein prenyltransferases have been reported. Protein farnesyltransferase catalyzes condensation of FPP and the cysteine residue of a CAAX sequence at the C-terminal region of precursor proteins (C, cysteine; A, aliphatic amino acid; X, methionine or serine). Protein geranylglyceranyltransferase I catalyzes a similar condensation of GGPP and the cysteine residue of a CAAX sequence of

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Abbreviations: IPP, isopentenyl diphosphate; DMAPP, dimethylallyl diphosphate; GPP, geranyl diphosphate; FPP, farnesyl diphosphate; GGPP, geranylglyceranyl diphosphate; PP, diphosphate; SDS-PAGE, sodium dodecylsulfate polyacrylamide gel electrophoresis; PMSF, phenylmethanesulfonyl fluoride.
precursor proteins (X, leucine or phenylalanine). Protein geranylgeranyltransferase II catalyzes the condensation of GGPP and one or both of the cysteine residues of a CXC or an XXCC sequence at the C-terminal region of precursor proteins. Concerning the biosynthesis of lipid precursors for these protein prenyltransferases, FPP, which has been generally accepted to be the common intermediate occupying the branch point in the biosynthetic pathways to cholesterol, dolichol, ubiquinone and Heme a, is supplied from DMAPP by the action of FPP synthase, the most abundant and widely occurring prenyltransferase in mammalian tissues. On the other hand, the biosynthesis of GGPP, the carbon chain length of which is longer by one isoprene unit than that of FPP, is not well understood. Reed & Rilling [10] have reported that crystalline FPP synthase from avian liver has a poor but significant ability to synthesize GGPP from FPP and IPP. We have separated GGPP synthase from FPP synthase by ion exchange chromatography followed by hydroxylapatite chromatography of crude extracts from pig liver [11]. Recently we have also reported separation of the two enzymes by chromatography of crude extracts from rat liver [12] and from bovine brain [13]. However, the farnesyl-transferring activity of the partially purified GGPP synthase free from FPP synthase was too low for the enzyme to be characterized.

To gain better understanding of the biosynthetic pathway to GGPP from DMAPP, the first starting allylic primer in the isoprenoid biosynthetic pathway, it is of importance to elucidate the relationship between FPP synthase and GGPP synthase. It is also of interest to see whether the level of GGPP as well as that of FPP in cells is controlled, as the number of geranylgeranylated proteins has been shown to be larger than that of farnesylated proteins [14–16]. Since the characterization of GGPP synthase has been hampered by the difficulty in purifying this enzyme by a series of combinations of conventional chromatographies, we tried to purify it in a one step procedure by affinity chromatography using an FPP analog as a ligand. This approach enabled us to purify GGPP synthase to homogeneity and to characterize the enzyme. In this report we describe these results.

### EXPERIMENTAL PROCEDURES

**Materials.** [1,14]CJPP (spec. act. 52 Ci/mol) was obtained from Amersham Corp. DMAPP, GPP, FPP, E,E-GGPP, Z,E,E-GGPP, and 2-(di-methylamino)ethyl-PP were prepared according to Davison et al. [17]. 3-Azageranylgeranyl-PP was provided by Prof. R.M. Coates, Department of Chemistry, University of Illinois, Urbana, IL. [U.S.A.]. Bovine brains were obtained from a local slaughterhouse. Affigel 10 agarose beads were purchased from Bio-Rad Laboratory. Mono Q, Superose 12 HR 10/30, and Superdex 200 10/30 columns were obtained from Pharmacia-LKB. Silica gel 60 thin-layer plates were obtained from Merck. Reverse-phase LKC-18 thin-layer plates were purchased from Whatman. All other chemicals were of reagent grade.

**Preparation of affinity gels.** Two kinds of ligands (see Fig. 1), O-(6-amino-1-hexyl)-P-geranyl methyl phosphonophosphate and O-(6-amino-1-hexyl)-P-farnesyl methyl phosphonophosphate, were prepared according to Bartlett et al. [18]. These ligands were almost pure as judged by H NMR spectrometry and by silica-gel thin-layer chromatography (RF: 0.13 and 0.17 for the geranyl methyl ligand and the farnesyl methyl ligand, respectively; eluent: 20% aqueous CH3CN containing 0.5% conc. NH4OH). Each ligand was coupled to Affigel 10 according to instructions of Bio-Rad company and the method of Bartlett et al. [18]. The affinity gels were stored at 4°C in 10 mM Pipes buffer (pH 7.0) containing 1 mM MgCl2, 10 mM 2-mercaptoethanol and 0.25 mM sodium azide until use.

**Enzyme purification.** Bovine brain was immersed in two volumes of 50 mM Tris/HCl buffer (pH 7.5) containing 1 mM EDTA, 1 mM EGTA, 0.1 mM leupeptin and 0.2 mM PMSF, cut into small pieces with scissors, and homogenized (five strokes) in a Teflon glass homogenizer. The homogenate was centrifuged at 10000 x g for 30 min and at 100000 x g for 2.5 h. For purification of GGPP synthase, the resulting supernatant was brought to 0.4 saturation with solid ammonium sulfate, stirred for 30 min, and centrifuged at 10000 x g for 15 min. The obtained supernatant was brought to 0.6 saturation with solid ammonium sulfate,
stirred for 30 min, and then centrifuged at 10000 × g for 15 min. The precipitate was dissolved in a small volume of standard buffer (10 mM Pipes, pH 7.0, 1 mM MgCl₂, and 10 mM 2-mercaptoethanol). The crude enzyme was dialyzed against standard buffer containing 0.1 M KCl and 1 mM sodium pyrophosphate. The dialysate was filtered through a membrane (DINMIC-25, 0.45 μm). The filtrate was diluted to 10–20 mg/ml with the same buffer as described above and loaded onto a farnesylmethylaffinity gel column (0.8 cm × 12 cm), and the column was eluted at a flow rate of 6 ml/h. A pre-column located immediately before the affinity column contained 5 ml of Affigel 10 agarose derivatized with 2-aminoethanol. The farnesylmethyl affinity gel column was then washed with standard buffer containing 0.1 M KCl and 1 mM sodium pyrophosphate until the absorbance at 280 nm came back to the base line. Subsequent elution steps were carried out at a flow rate of 30 ml/h. The column was washed with standard buffer containing 0.2% (w/v) octylglucoside to remove non-specifically bound proteins, followed by washing with standard buffer. GGPP synthase was eluted with a linear gradient of FPP (0–500 μM) in standard buffer. The fractions showing the enzyme activity were combined, concentrated in a Centricon-3 concentrator apparatus, washed with standard buffer three times to remove FPP and stored at −80°C in the presence of 0.2% octylglucoside until use.

For purification of FPP synthase, the supernatant obtained by centrifugation at 100000 × g of the homogenate from bovine brain was brought to 3% saturation with solid ammonium sulfate, stirred for 30 min, and centrifuged at 10000 × g for 15 min. The supernatant was brought to 4% saturation with solid ammonium sulfate, stirred for 30 min, and centrifuged at 10000 × g for 15 min. The precipitate was dissolved in a small volume of standard buffer and dialyzed against the same buffer. The dialysate was then filtered through the membrane (DINMIC-25, 0.45 μm), and the filtrate was diluted to 10–20 mg/ml with standard buffer and loaded onto a geranyl methyl affinity gel column (0.8 cm × 12 cm). Subsequent elution conditions were the same as described by Bartlett et al. [18].

Prenyltransferase assay and product analysis. The standard assay mixture contained, in a final volume of 25 μl, 50 mM phosphate buffer (pH 7.0), 5 mM MgCl₂, 2 mM dithiothreitol, 0.1% (w/v) bovine serum albumin, 0.8% (w/v) octylglucoside, 25 μM alliacyl diphosphate, 20 μM [1-14C]IPP, 20 μM 2-(dimethylamino)ethyl-PP and a suitable amount of enzyme protein. The mixture was preincubated in the absence of alliacyl diphosphate and [1-14C]IPP at 37°C for 5 min to inhibit the action of IPP isomerase, then the substrates were added and the incubation continued at 37°C for 30 min. When a purified enzyme was used, the preincubation in the presence of 2-(dimethylamino)ethyl-PP was omitted. The enzymatic reactions were stopped by the addition of a mixture (0.3 ml) of conc. HCl:CH₃OH (1:4, v/v). The mixture was kept at 37°C for 15 min and extracted with hexane. The radioactivity in the hexane extracts was measured by scintillation counting.

For product analysis, the enzymatic reaction products were extracted with 1-butanol and treated with 1-phosphate at 37°C for at least 5 h according to Fujii et al. [19]. The liberated products were extracted with hexane. The hexane soluble products were analyzed by reverse-phase LKC-18 thin-layer chromatography in acetone:water (7:1, v/v). The positions of authentic standards were visualized with iodine vapor. For autoradiography, the thin-layer plates were exposed on a Fuji imaging plate at room temperature for one day. The exposed imaging plate was analyzed with a Fuji BAS 2000 Bioimage analyzer.

Molecular mass of native enzyme. A Superose 12 column and a Superdex 200 column were eluted with standard buffer containing 0.1 M NaCl and 5 μM FPP at a rate of 0.5 ml/min. Protein standards were β-galactosidase (465 kDa), immunoglobulin G (150 kDa), Fab fragment from immunoglobulin G (50 kDa), and myoglobin (17 kDa). Fractions were collected and assayed for GGPP synthase activity.

RESULTS

Purification of geranylgeranyl-PP synthase

For purification of the enzyme from bovine brain we prepared two kinds of affinity gels, geranyl methyl affinity gel and farnesylmethyl affinity gel. We first tried to use the geranyl methyl affinity gel, which has been reported to be
effective in purifying FPP synthase [18]. When the 0.4–0.6 ammonium sulfate fraction containing geranyl-transferring and farnesyl-transferring activity was loaded onto the column under the conditions reported in [18], neither activity was observed in the flow-through fraction, but both activities were observed in the fraction eluted by inorganic pyrophosphate (1 mM). Next, we tried to use the farnesylmethyl affinity gel. Neither geranyl-transferring nor farnesyl-transferring activity of the 0.4–0.6 ammonium sulfate fraction was observed in the flow-through fraction, like in the case of the geranyl methyl affinity gel. When the column was eluted with inorganic pyrophosphate (1 mM), an enzyme with geranyl-transferring activity was released from the gel. Another enzyme with farnesyl-transferring activity was recovered with a linear gradient of farnesyliPP (0–500 μM). These results suggested that GGPP synthase can bind to the farnesylmethyl affinity gel in the presence of inorganic pyrophosphate (1 mM) and that the bound enzyme can be released from the gel by FPP. Since SDS-polyacrylamide gel electrophoresis of the GGPP synthase fraction thus obtained showed several protein bands, we next tried to establish better conditions to remove the proteins which were non-specifically bound to the gel. Figure 2 shows a typical farnesylmethyl affinity chromatogram of the 0.4–0.6 ammonium sulfate fraction. When individual fractions were assayed with a combination of FPP and [1,14C]IPP in the presence of 2-(dimethylamino)ethyl-PP, two activity peaks were observed. Product analysis revealed that the flow-
Fig. 3. SDS-Polyacrylamide gel electrophoresis of fractions ranging from 142 to 149 of farnesylmethyl affinity gel column chromatography. The gel was stained with Coomassie Brilliant Blue.

through fractions corresponding to the first peak contained IPP isomerase and FPP synthase and that the fractions corresponding to the second peak contained GGPP synthase. Next, the fractions (no. 142–149) corresponding to GGPP synthase were analyzed by SDS-polyacrylamide gel electrophoresis (Fig. 3). When the gel was stained with Coomassie Brilliant Blue, a protein band corresponding to the molecular mass of 37.5 kDa was observed in parallel with farnesyl-transferring activity of GGPP synthase. To confirm whether this protein band corresponded in fact to the latter enzyme, we further carried out Mono Q chromatography of the GGPP synthase fraction obtained by farnesylmethyl affinity chromatography. The active enzyme eluted at the concentration of 0.23 M NaCl was associated with the protein band corresponding to the molecular mass of 37.5 kDa. SDS-Polyacrylamide gel electrophoresis of the purified enzyme gave practically a single band as shown in Fig. 4. The GGPP synthase was about 2000 fold purified with a specific activity of 98 nmol/min per mg protein as summarized in Table 1. We also tried to purify FPP synthase from bovine brain by geranyl methyl affinity chromatography. In this case, the 0.3–0.4 ammonium sulfate fraction containing geranyl-transferring activity but no farnesyl-transferring activity was used. The FPP synthase was purified about 1600 fold with a specific activity of 464 nmol/min per mg protein.

Molecular mass of native enzyme

The GGPP synthase fraction purified by farnesylmethyl affinity chromatography was subjected to Superose 12 gel filtration. Molecular mass of the active enzyme was estimated to be about 150.0 kDa. In the case of Superdex 200 gel filtration, the eluted active enzyme had a molecular mass of 190 kDa. Either active fraction showed a protein band corresponding to 37.5 kDa on SDS-polyacrylamide gel electrophoresis. We also carried out Superose 12 gel filtration of the FPP synthase purified by geranylmethyl affinity chromatography. In this case, the apparent molecular mass of the enzyme was 80 kDa, while its SDS-polyacrylamide gel electrophoresis showed a single protein band corresponding to 40 kDa. These results indicate that the FPP synthase (80 kDa) is composed of two similar or identical subunits, whereas the GGPP synthase (molecular mass 150–190 kDa) is composed of four or five subunits of similar or identical size.

Properties of geranylgeranyl-PP synthase

Figure 5A and 5B show a reverse-phase and a normal phase silica-gel thin-layer chromatogram, respectively, of the hydrolysates obtained by acid phosphatase treatment of the products derived from [1-14C]IPP and DMAPP, GPP, FPP, E,E,E-GGPP, or Z,E,E-GGPP. When DMAPP was the allylic substrate (lane 2), C20
Marker
<kDa>
97.4
66.2
42.7
31.0
21.5
14.4
Lane 1 2 3

GGPP was the major product. In the case of the reaction with GPP (lane 3), both FPP and GGPP were formed. When FPP (lane 4) was used as allylic primer, GGPP was practically the only product formed, and no product was of length exceeding C20. No product was formed in the reaction with E,E,E-GGPP or its stereoisomer, Z,E,E-GGPP (not shown). To study the variability of products of the enzyme, we quantitatively analyzed the distribution of farnesyl and geranylgeranyl products on the reverse-phase thin-layer plate of Fig. 5A (Table 2). The ratio of reaction velocities for formation of GGPP from DMAPP, GPP, and FPP was 0.004:1.045:1. We also examined the dependence on the concentration of allylic substrates. The $K_m$ value for DMAPP, GPP, and FPP were estimated from Lineweaver-Burk plots to be 33 μM, 0.80 μM,

**Table 1**

Purification of geranylgeranyl-PP synthase by farnesylmethyl affinity gel column chromatography.

<table>
<thead>
<tr>
<th>Step</th>
<th>Total activity (nmol/min)</th>
<th>Protein (mg)</th>
<th>Specific activity (nmol/min per mg protein)</th>
<th>Yield (%)</th>
<th>Purification (factor)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.4-0.6 (NH₄)₂SO₄ sat.</td>
<td>55.8</td>
<td>1156.8</td>
<td>0.0482</td>
<td>100</td>
<td>1</td>
</tr>
<tr>
<td>F-Affinity</td>
<td>12.8</td>
<td>0.190</td>
<td>67.4</td>
<td>22.9</td>
<td>1398</td>
</tr>
<tr>
<td>Mono Q</td>
<td>4.8</td>
<td>0.049</td>
<td>98.0</td>
<td>8.6</td>
<td>2033</td>
</tr>
</tbody>
</table>

Fig. 4. SDS-Polyacrylamide gel electrophoresis of purified geranylgeranyl-PP synthase.

The gel was stained with Coomassie Brilliant Blue. Lane 1, molecular mass markers; lane 2, the enzyme purified on farnesylmethyl affinity gel; lane 3, the enzyme purified by Mono Q after farnesylmethyl affinity gel chromatography.
Fig. 5. Reverse-phase thin-layer chromatogram (A) and normal-phase thin-layer chromatogram (B) of hydrolysates obtained by acid phosphatase treatment of the products of enzymatic reactions.

Reverse-phase and normal-phase thin-layer chromatographies were performed in solvent systems of acetone:water (9:1, v/v) and benzene:ethyl acetate (7:1, v/v), respectively. The developed plates were analyzed with a Fuji BAS 2000 Imaging analyzer. Incubations were conducted with [1-14C]isopentenyl-PP alone (lane 1) and with [1-14C]isopentenyl-PP and dimethylallyl-PP (lane 2); [1-14C]isopentenyl-PP and geranyl-PP (lane 3); [1-14C]isopentenyl-PP and farnesyl-PP (lane 4). S.F., solvent front; Ori, origin; GOH, geraniol; FOH, farnesol; GGOH, geranylgeraniol.
Table 2

Distribution of farnesyl and geranylgeranyl products.

Beta-ray intensity of the radioactivity corresponding to farnesol and geranylgeraniol on the reverse-phase plate of Fig. 5A was measured with a Fuji BAS 2000 Biomage analyzer. The figures in parentheses indicate the relative velocities for the C₅→C₂₀, C₁₀→C₂₀, and C₁₅→C₂₀ steps of the reaction calculated on the assumption that the radioactivities of GGPP formed from [1-¹⁴C]isopentenyl-PP with dimethylallyl-PP and with geranyl-PP relative to that of geranylgeranyl-PP formed from [1-¹⁴C]isopentenyl-PP with farnesyl-PP are 3 and 2, respectively.

<table>
<thead>
<tr>
<th>Allylic substrate</th>
<th>Farnesyl</th>
<th>Geranylgeraniol</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dimethylallyl-PP</td>
<td>0 (0.00)</td>
<td>26 (0.034)</td>
</tr>
<tr>
<td>Geranyl-PP</td>
<td>1778 (0.74)</td>
<td>694 (0.145)</td>
</tr>
<tr>
<td>Farnesyl-PP</td>
<td>0 (0.00)</td>
<td>2389 (1.00)</td>
</tr>
</tbody>
</table>

and 0.74 μM, respectively. The Kᵥ value for IPP in the reaction with FPP was 2 μM. These results indicate that FPP is the best allylic substrate, suggesting that the GGPP synthase essentially catalyzes a single reaction of C₁₅→C₂₀. The enzyme required a divalent metal ion for maximum activity (Fig. 6). Mg²⁺ was much more effective at 1 mM than Mn²⁺ or Zn²⁺. The pI for the enzyme was estimated to be 6.2 by isoelectric focusing gel electrophoresis. The enzyme was activated 1.2 fold by 0.8% (w/v) octylglucoside.

DISCUSSION

Geranylgeranyl-PP synthase and farnesyl-PP synthase from bovine brain, purified to homogeneity, were found to be different proteins. The two enzymes differ in substrate specificity and subunit composition. On the basis of the results obtained it is concluded that, in the biosynthetic pathway to GGPP, two prenyltransferases are engaged: FPP synthase catalyzing the reaction C₅→C₁₅ and GGPP synthase catalyzing the reaction C₁₅→C₂₀.

It should be noted that GGPP synthase has the capability of catalyzing the formation of FPP and GGPP from IPP in the presence of IPP. Though the geranyl-transferring activity is lower than the farnesyl-transferring activity, GPP is accepted by GGPP synthase with a Kᵥ value (0.80 μM) similar to that for FPP (0.74 μM). This would imply that FPP and GGPP, lipid precursors for the biosynthesis of various prenylated proteins, can be synthesized from GPP in the presence of IPP by the action of GGPP synthase alone without participation of FPP synthase. However, this implication seems unlikely, since it has been accepted at present that GPP has no other way of being supplied than as the intermediate of the reaction of DMAPP and IPP catalyzed by FPP synthase.

In a previous report [11] we demonstrated that a partially purified preparation of GGPP

Fig. 6. Effect of metal ions on geranylgeranyl-PP synthase.
The incubation mixtures contained [1-¹⁴C]isopentenyl-PP and farnesyl-PP in the presence of varied concentrations of MgCl₂ (•), MnCl₂ (■), or ZnCl₂ (△).
Table 3
Substrate specificity of geranylgeranyl-PP synthase.

For the estimation of $K_m$ values, incubations were conducted in the presence of a fixed concentration of [1-14C]cloisopentenyl-PP (20 $\mu$M) and varied concentrations of allylic diphosphate. $V_{\max}$ values were calculated using Lineweaver-Burk analysis. $V_{\max}$ shows the relative reaction velocity with a combination of [1-14C]cloisopentenyl-PP (20 $\mu$M) and allylic diphosphate (25 $\mu$M). The formation of geranylgeranyl-PP was analyzed as described for Fig. 5A and Table 2.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>$K_m$ (µM)</th>
<th>$V_{\max}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dimethylallyl-PP</td>
<td>33</td>
<td>0.004</td>
</tr>
<tr>
<td>Geranyl-PP</td>
<td>0.80</td>
<td>0.145</td>
</tr>
<tr>
<td>Farnesyl-PP</td>
<td>0.74</td>
<td>1.00</td>
</tr>
</tbody>
</table>

synthase from pig liver had a molecular mass of 300 kDa. Similarly, we reported a much larger value for the molecular mass (120 kDa) of partially purified GGPP synthase from rat liver [12] than the values generally found in prenyltransferases i.e. in FPP synthase. These observations led us to the supposition that mammalian GGPP synthase occurs in a complex form composed of several different or identical subunits. The present study showed that a highly purified preparation of GGPP synthase occurred, in fact, in an oligomeric form with an apparent molecular mass of 150-195 kDa, but on SDS-polyacrylamide gel electrophoresis the enzyme gave a homogenous protein band. Alternatively, the GGPP synthase could be composed of a smaller number of subunits but be quite asymmetrical in shape. The molecular mass of the enzyme monomer (37.5 kDa) is consistent with that of several GGPP synthases (30-40 kDa) from other sources, such as plants [20, 21], a fungus [22], and bacteria [23, 24], though these enzymes have been reported to occur in a dimeric form. Thus, it is very interesting that mammalian geranylgeranyl-PP synthase has unique properties with respect to the organization of subunits.

REFERENCES


