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Human Geranylgeranyl Diphosphate Synthase is an Octamer in Solution

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A recombinant geranylgeranyl diphosphate synthase (GGPS) was analysed to be a mixture of octamer, hexamer and dimer by gel filtration using a Superdex 200 column followed by the blue native polyacrylamide gel electrophoresis. The hexamer and dimer were each converted to an octamer by treating with dithiothreitol (DTT). When the recombinant GGPS was preliminarily treated with DTT and similarly analysed, octamer was predominantly detected with a trace amount of hexamer. The octameric form of GGPS was also supported by the cross-linking experiments with bis(sulfosuccinimidyl) suberate. The GGPS in an octameric form was active with a combination of farnesyl diphosphate and [1-^{14}C]isopentenyl diphosphate. These results indicate that the active form of GGPS in the solution is an octamer rather than hexamer or dimer.

Key words: cross-linking, geranylgeranyl diphosphate synthase, isoprenylation, prenyltransferase, structure and activity.

Abbreviations: BS^3, bis(sulfosuccinimidyl) suberate; DTT, dithiothreitol; FPS, farnesyl diphosphate synthase; GGPS, geranylgeranyl diphosphate synthase; PAGE, polyacrylamide gel electrophoresis; PP, diphosphate; TCEP, tris(2-carboxyethyl)phosphine.

Farnesyl diphosphate synthase (FPS) and geranylgeranyl diphosphate synthase (GGPS) are both classified into a *trans*-type prenyltransferase family, catalysing the fundamental chain elongation reaction between allylic diphosphate and isopentenyl diphosphate (isopentenyl-PP). The former resides at a key branch point of the mevalonate pathway and produces precursors (farnesyl diphosphate, farnesyl-PP) for all isoprenoids (*1*). On the other hand, the latter produces geranylgeranyl diphosphate (geranylgeranyl-PP) from farnesyl-PP. Geranylgeranyl-PP serves as a precursor for the geranylgeranylation of Rho and Rac involved in cytoskeletal organization and of Rab involved in vesicular transportation, and the lipid modifications of those proteins are essential for their function. Geranylgeranyl-PP has also been known to act as a negative regulator for DNA binding of liver X receptor (*2*).

Recently, it has been reported that the nitrogen-containing bisphosphonates, used to treat disorders characterized by bone resorption such as osteoporosis, Paget's disease or multiple myeloma, inhibit FPS but not GGPS (*3–5*). Since it is expected that both enzymes are similar to each other in prenyl-transfer active sites, it is important to clarify how bisphosphonates are recognized by these two enzymes. In the case of human FPS, the structure was determined to be dimer (*6*) and the molecular mechanism of nitrogen-containing bisphosphonates was also deduced. On the other hand, mammalian GGPSs have been reported to behave as an oligomer on gel filtration (*7*, *8*). In the case of human GGPS (*9–11*), the structure was also determined to be an oligomer (*9*) by the similar gel filtration method, and none of the nitrogen-containing bisphosphonates that inhibit FPS is effective (*12*). The structural difference between FPS and GGPS might be related to the conformation of each active site. However, it is still unclear whether GGPS forms a dimer like FPS and further assembled to form an oligomer.

In the present study, we analysed recombinant human GGPS by the methods of gel filtration, blue native polyacrylamide gel electrophoresis (blue native-PAGE), and chemical cross-linking, and now report that human GGPS in solution is an octamer comprised of four dimers.

EXPERIMENTAL PROCEDURES

Expression of Human GGPS in Escherichia coli Cells—The expression vector, pET15b-hGGPS was prepared as described in our previous report (*12*) and used for the expression of histidine-tagged recombinant human GGPS. The *E. coli* BL21(DE3) cells transformed with the pET plasmid were grown at 37°C to an A_{600} of 0.6. Isopropyl 1-thio-β-galactopyranoside was added to a final concentration of 1 mM, and then the cells were cultured at 37°C for 4 h.

Purification of Human GGPS—The cells were collected and suspended in lysis buffer (20 mM Tris–HCl pH 7.9, 5 mM ethylenediaminetetraacetic acid and 1 mM phenylmethylsulfonyl fluoride). The cell suspension was sonicated on ice and centrifuged at 12,000 × *g* for 10 min. The supernatant was passed through a 0.22 μm filter prior to application to a HiTrap Chelating HP column

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(0.7 cm × 2.5 cm, 1 ml) (Amersham-Pharmacia Biotech) pre-charged with nickel and pre-equilibrated in binding buffer (20 mM Tris–HCl pH 7.9 and 5 mM imidazole). The column was washed with wash buffer (20 mM Tris–HCl pH 7.9, 0.5 M NaCl and 60 mM imidazole). The proteins were eluted with elute buffer (20 mM Tris–HCl pH 7.9, 0.5 M NaCl and 1 M imidazole). The protein-containing fractions were transferred to Amicon Ultra-4 10,000 NMWL (MILLIPORE) and concentrated by centrifugation at 3,000 × *g* for 10 min using a swinging bucket rotor. The proteins were further treated with thrombin from bovine plasma (WAKO) in cleavage buffer (20 mM Tris–HCl pH 8.4, 0.15 M NaCl, 2.5 mM $CaCl_2$) at 4°C overnight followed by the removal of histidine-tagged protein by passing the digest over a HiTrap Chelating HP column. The unbound fractions were pooled. The untagged proteins were analysed by sodium dodecyl sulfate (SDS) PAGE and stored at 4°C before use.

Gel Filtration and Blue Native-PAGE—Gel filtration was performed using a Superdex 200 column (16/600) equilibrated with 50 mM potassium phosphate buffer pH 7.0 containing 0.15 M NaCl at a flow rate of 1 ml/min. The protein elution was monitored at 280 nm. The column was calibrated using standard proteins: ferritin (440 kDa), catalase (232 kDa), albumin (67 kDa), ovalbumin (43 kDa) and chymotrypsinogen (25 kDa). Blue native-PAGE (Invitrogen) was performed according to the manufacturer's procedure (*13*, *14*). The molecular standards were apoferritin band 1 (720 kDa), apoferritin band 2 (480 kDa), beta-phycoerythrin (242 kDa), lactate dehydrogenase (146 kDa), bovine serum albumin (66 kDa) and soybean trypsin inhibitor (20 kDa). Proteins were detected by silver staining using a commercially available kit (Daiichi Pure Chemicals).

Prenyltransferase Assay and Product Analysis—The standard assay mixture contained, in a final volume of 25 µl, 50 mM potassium phosphate buffer pH 7.0, 5 mM $MgCl_2$, 2 mM dithiothreitol (DTT), 25 µM allylic diphosphate, 20 µM [1-^{14}C]isopentenyl-PP (s.a. 59 µCi/µmol) and a suitable amount of enzyme. The mixture was incubated at 37°C for 15 min, and the pH was lowered by the addition of 75 µl of HCl/MeOH [1/4 (v/v)] to complete the hydrolysis of allylic products. After 15 min incubation at 37°C, the mixture was neutralized by the addition of 37.5 µl of 6N NaOH. Liberated prenyl alcohols in the mixture were then extracted with 150 µl of hexane, and an aliquot (75 µl) was transferred to a scintillation vial for radioactivity counting with liquid scintillation counter (BECKMAN LS-6500).

Chemical Cross-linking of GGPS—GGPS proteins were cross-linked with disuccinimidyl suberate (DSS, spacer arm length 11.4 Å), bis(sulfosuccinimidyl) suberate (BS^3, spacer arm length 11.4 Å), or ethylene glycolbis(sulfosuccinimidylsuccinate) (Sulfo-EGS, spacer arm length 16.1 Å). Cross-linkers were added to the proteins (8 µM) at various concentration of 0.2 to 40 mM in the absence or presence of 5 mM $MgCl_2$, 25 µM farnesyl-PP, 2 mM DTT, and/or 1 mM tris(2-carboxyethyl)phosphine (TCEP), and the mixtures were shaken at 4 or 20°C for 0–12 h and quenched by the addition of 250 mM Tris–HCl pH 7.5. The cross-linked products were analysed by SDS-PAGE and detected by silver staining. Two kinds of molecular standard mixtures were used. One standard mixture (Fermentas, SM0661) contains proteins corresponding to 200, 150, 120, 100, 85, 70, 60, 50 and 40 kDa. The other standard mixture (Invitrogen, LC5699) contains proteins corresponding to 460, 268, 238, 171, 117, 71, 55 and 41 kDa.

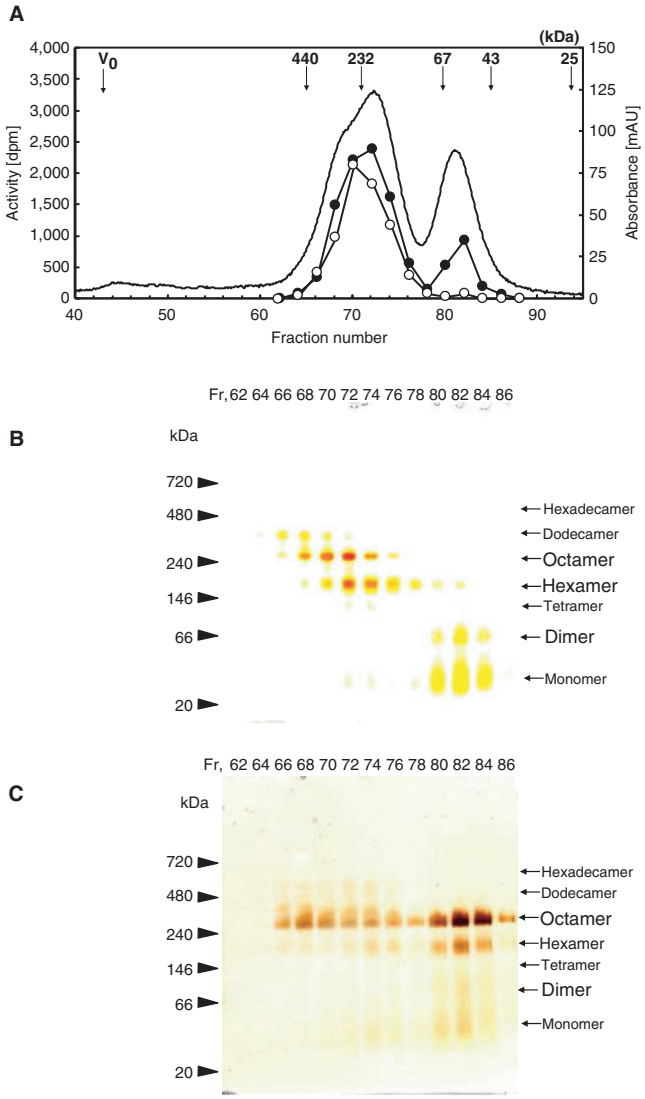

Fig. 1. **Chromatographic behavior of human recombinant GGPS.** (A) Gel filtration of GGPS. Thrombin-cleaved untagged GGPS (0.5 mg) was kept overnight at 4°C in 1.0 ml of 20 mM Tris–HCl buffer (pH 7.0) containing 0.5 M NaCl and injected onto a Superdex 200 column. Fractions (1 ml) were collected. The solid line represents absorbance at 280 nm. The enzyme activity was assayed in the absence (open circles) and presence (closed circles) of DTT as described in Experimental procedures section. (B) Blue native-PAGE of fractions (62–86) shown in (A). GGPS proteins were detected by silver staining. (C) Blue native-PAGE of fractions (62–86) shown in (A) after treatment with DTT. The fractions were kept at 4°C in the presence of 2 mM DTT overnight and analysed. GGPS proteins were detected by silver staining.

RESULTS AND DISCUSSION

The untagged GGPS (303 amino acids, 35.2 kDa) obtained by the treatment of expressed histidine-tagged GGPS with thrombin was kept overnight in Tris–HCl buffer pH 7.0 and subjected to the gel filtration using a Superdex 200 column. As shown in Fig. 1A, two peaks were observed. The first and second peaks with elution volumes corresponding to a molecular mass of about 200–300 and 70 kDa were compatible with octameric/hexameric and dimeric species, respectively. When each fraction was subjected to blue native-PAGE, octameric (281 kDa) and hexameric (210 kDa) forms were detected in fractions corresponding to the first peak, whereas dimeric (70 kDa) and monomeric (35.2 kDa) forms were detected in fractions corresponding to the second peak (Fig. 1B). Dodecameric forms were a little detected in fractions (66–70). When each fraction was assayed with the same combination of farnesyl-PP and [1-^{14}C]isopentenyl-PP in the absence of DTT, the octamer and hexamer-containing fractions were active, but the dimer-containing fraction was inactive (Fig. 1A). However, when each fraction was assayed with the same combination of farnesyl-PP and [1-^{14}C]isopentenyl-PP in the presence of DTT, the octamer and hexamer-containing fractions showed a little enhanced activity and the dimer-containing fraction showed activity. These results suggested that GGPS forms octamer (four dimers), hexamer (three dimers) and dimer in conditions at pH 7.0 and that these three forms each contain oxidized GGPS subunits. Considering the occurrence of three cysteine residues per one GGPS subunit, we treated each fraction (62–86) ranging from oligomer to dimer fractions with DTT overnight and subjected them to blue native-PAGE. As shown in Fig. 1C, octameric forms were detected in all the fractions. The results indicate the occurrence of equilibrium between octamer and hexamer or dimer through oxidation and reduction of GGPS. Further, to understand the reduced GGPS forms, we pre-treated the recombinant GGPS with DTT prior to gel filtration (Fig. 2A). As expected, one peak corresponding to a molecular mass of 281 kDa was observed with enzyme activity irrespective of the absence or presence of DTT, and the major form was octamer, though a trace amount of hexamer was detected (Fig. 2B). Also, hexadecameric forms were slightly detected in fractions (62–64).

In cross-linking experiments, at first, we tried to use histidine-tagged GGPS because it also behaved like an octamer on gel filtration. In the case of DSS, which is water-insoluble with a space arm length of 11.4 Å, two products were detected, corresponding to monomer and dimer. In case of BS^3, which is water-soluble with the same spacer arm length as DSS, not only the similar two products but also another product corresponding to hexamer was detected (data not shown). We next tried to use untagged GGPS and also Sulfo-EGS, which is water-soluble with a spacer arm length of 16.1 Å as well as BS^3. In the case of Sulfo-EGS, which has a longer space arm than BS^3, products corresponding to monomer, dimer and oligomer were detected by SDS-PAGE, but the detectable amount of oligomer was extremely small compared to the case using BS^3 (data not shown). Fig. 3 shows a typical SDS-PAGE of products treated

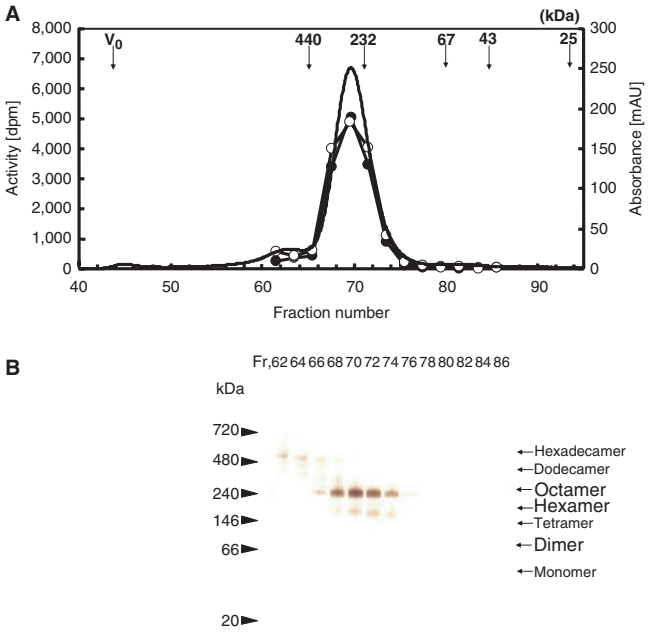

Fig. 2. **Chromatographic behaviour of DTT-treated human recombinant GGPS.** (A) Gel filtration of GGPS treated with DTT. Thrombin-cleaved untagged GGPS (2 mg) was treated as shown in (A) of Fig. 1 in the presence of 2 mM DTT and applied to the same Superdex 200 column. Fractions (1 ml) were collected. The solid lines represent absorbance at 280 nm. The enzyme activity was assayed in the absence (open circles) and presence (closed circles) of DTT as described in Experimental procedures section. (B) Blue native-PAGE of fractions (62–86) shown in (A). GGPS proteins were detected by silver staining.

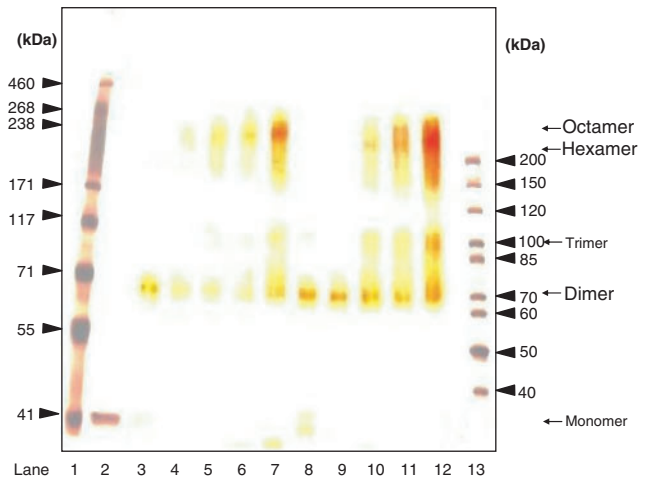

Fig. 3. **Human recombinant GGPS cross-linked with BS^3.** Untagged GGPS (100 ng) was treated with 20 mM BS^3 at 20°C as described in Experimental procedures section, and cross-linked products were analysed by SDS-PAGE (4–20%) followed by silver staining. Lane 1 and 13, Molecular mass standards; lane 2, untreated GGPS; Lane 3–7 and 8–12, GGPS treated with BS^3 in the presence of 2 mM DTT and of 1 mM TCEP, respectively, for 0, 5, 15, 30 and 60 min.

with BS^3. Dimeric products were detected as expected and trimeric-like products (lanes 7, 10, 11 and 12) were also detected. Monomeric products detected predominantly in cross-linking experiments (all lanes) migrated much faster than the untreated monomer (lane 2) (not shown in Fig. 3). Oligomeric products corresponding to a molecular mass of about 250 kDa were detected in a time-dependent manner in presence of DTT (lane 3–7) or TCEP (lane 8–12). The major oligomer band (lane 7) coincided with the upper band of the two that overlapped each other (lane 11). Considering that molecular masses of untreated octamer and hexamer are calculated to be 281 and 210 kDa, respectively, we concluded that the maximally cross-linked products are octamer.

In the present study, we analysed the human GGPS structure by the methods of gel filtration, blue native-PAGE and chemical cross-linking. Several lines of evidence show that the major active form of human GGPS in solution is octamer comprising of four dimers. Although hexameric and dimeric forms were detected on gel filtration, these seem to be derived from reduced octameric forms by spontaneous oxidation because the hexameric and dimeric forms were convertible to octameric forms by treating with DTT (Fig. 1C). We rechromatographed each of the octamer- and hexamer-enriched fractions and assayed activities and protein concentrations of each purified fraction. The ratio of specific activity between octameric and hexameric enzymes was relativity estimated to be 1.00: 0.56 (data not shown). It would be acceptable that there is equilibrium between octamer and hexamer through oxidization and reduction of cysteine residues (equilibrium 1). The dodecameric form detected in a small amount as shown in Fig. 1B might consist of two oxidized hexamer under equilibrium 1. Also, we speculate another equilibrium between octamer and hexamer of reduced GGPS forms (equilibrium 2) because the small amount of hexamer was always detected as shown in Fig. 2B. The hexadecameric form detected in a small amount as shown in Fig. 2B might consist of two reduced octamers under equilibrium 2. The occurrence of hexameric forms under equilibrium 2 was recently supported by the structure of crystal GGPS reported by Kavanagh *et al.* (*15*). They showed that human GGPS is a hexamer comprised of three dimers and that the three dimers join together to form a propeller-bladed hexameric molecule with a mass of about 210 kDa. They also showed that GGPS behaved as hexamer on gel filtration. So, according to their chromatographic conditions we also tried to perform gel filtration for our prepared recombinant GGPS. Blue native-PAGE, however, revealed the presence of octamer (data not shown). Since all the cysteine moieties in crystal are analysed to be in reduced forms, the hexamers in equilibrium 2 would be crystallized. It is probable that GGPS is an oligomer consisting of dimer unit, that the inter-dimer regions of crystal hexamers are similar to those of hexamers shown in the present study, and that these regions are different from those of active octamers. It might be possible to assume that three of octamer species are assembled to form a putative tetracosamer as an intermediate and that the intermediate is rearranged to form four hexamer species. Alternatively, one octamer species might be equilibrated with hexamer species through an intermediate dimer species. In both cases, it is expected that the inter-dimer regions are considerably different between octameric and hexameric forms. This implies that the structure constituting active sites might differ between octamer in solution and hexamer in crystal. To understand the different effects of nitrogen-containing bisphosphonates on FPS and GGPS, further studies including X-ray structural analysis of active GGPS octamer and NMR analysis of GGPS in solution are necessary.

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