Interaction with the Small Subunit of Geranyl Diphosphate Synthase Modifies the Chain Length Specificity of Geranylgeranyl Diphosphate Synthase to Produce Geranyl Diphosphate*

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Geranyl diphosphate synthase belongs to a subgroup of prenyltransferases, including farnesyl diphosphate synthase and geranylgeranyl diphosphate synthase, that catalyzes the specific formation, from C\(_5\) units, of the respective C\(_{10}\), C\(_{15}\), and C\(_{20}\) precursors of monoterpenes, sesquiterpenes, and diterpenes. Unlike farnesyl diphosphate synthase and geranylgeranyl diphosphate synthase, which are homodimers, geranyl diphosphate synthase from *Mentha* is a heterotetramer in which the large subunit shares functional motifs and a high level of amino acid sequence identity (56–75%) with geranylgeranyl diphosphate synthases of plant origin. The small subunit, however, shares little sequence identity with other isoprenyl diphosphate synthases; yet it is absolutely required for geranyl diphosphate synthase catalysis. Coexpression in *Escherichia coli* of the *Mentha* geranyl diphosphate synthase small subunit with the phylogenetically distant geranylgeranyl diphosphate synthases from *Taxus canadensis* and *Abies grandis* yielded a functional hybrid heterodimer that generated geranyl diphosphate as product in each case. These results indicate that the geranyl diphosphate synthase small subunit is capable of modifying the chain length specificity of geranylgeranyl diphosphate synthase (but not, apparently, farnesyl diphosphate synthase) to favor the production of C\(_{15}\) chains. Comparison of the kinetic behavior of the parent prenyltransferases with that of the hybrid enzyme revealed that the hybrid possesses characteristics of both geranyl diphosphate synthase and geranylgeranyl diphosphate synthase.

A subgroup of isoprenyl diphosphate synthases, referred to as the “short-chain prenyltransferases,” consists of geranyl diphosphate (GPP; C\(_{15}\)) synthase, farnesyl diphosphate (FPP; C\(_{15}\)) synthase, and geranylgeranyl diphosphate (GGPP; C\(_{20}\)) synthase. These enzymes provide the acyclic branch point intermediates for the biosynthesis of numerous terpenoids, including monoterpenes, sesquiterpenes, diterpenes, triterpenes, tetraterpenes, and polyterpenes such as natural rubber. GGPP synthase and FPP synthase occur nearly ubiquitously in plants, animals, and bacteria (1). GPP synthase appears to be of much more limited distribution in nature, having been identified most frequently in essential oil (monoterpene)-producing plants (2). Most isoprenyl diphosphate synthases, including the short-chain prenyltransferases, catalyze the divergent metal ion-dependent 1'-4 condensation of isopentenyl diphosphate (IPP) with an allylic prenyl diphosphate co substrate (3), and they are distinguished by the specific chain length and double bond geometry at C\(_2\)–C\(_3\) of the prenyl diphosphate product generated (Fig. 1). Thus, GPP synthase catalyzes a single condensation of IPP with dimethylallyl diphosphate (DMAPP) to form, specifically, GPP (C\(_{15}\)). FPP synthase and GGPP synthase catalyze sequential condensations of IPP with an allyl primer (i.e. DMAPP, GPP, or FPP, as appropriate) to form the respective C\(_{15}\) and C\(_{20}\) elongation products. Reaction parameters, such as substrate concentration (4, 5) and metal ion cofactor (6), are known to modify chain length specificity of some prenyltransferases. Evaluation of the crystal structure of homodimeric avian FPP synthase (7), coupled with prenyltransferase sequence alignment data, has led to the directed mutagenesis of FPP synthase to alter the contour of the active site, thereby generating mutant enzymes that synthesize GGPP (8) or GPP (9, 10). A random chemical mutagenesis approach has also provided an altered FPP synthase capable of producing GGPP (11) and has yielded homodimeric GGPP synthase mutants capable of producing FPP (5) and polypropenols greater than C\(_{20}\) (12). This work eventually led to the direct mutagenesis of an archaeal GGPP synthase so as to produce FPP by the modified enzyme (13). These experiments clearly indicate that amino acid substitutions to alter the contour of the active site may either restrict or allow sequential elongation of the polyprenyl chain.

cDNAs encoding the large and small subunits of GPP synthase were isolated from a peppermint (*Mentha piperita*) oil gland library and were confirmed by functional coexpression of the heteromeric enzyme (14). Thus, GPP synthase, in which both subunits are absolutely required for prenyltransferase activity, is unlike both FPP synthases and GGPP synthases that are functional homodimers (1). The GPP synthase large subunit (GPPS.lsu) shows a high level of deduced amino acid sequence identity (56–75%) with GGPP synthases of plant origin.

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The abbreviations used are: GPP, geranyl diphosphate; FPP, farnesyl diphosphate; GGPP, geranylgeranyl diphosphate; IPP, isopentenyl diphosphate; DMAPP, dimethylallyl diphosphate; lsu, large subunit; ssu, small subunit; GPPS, geranyl diphosphate synthase; FPPS, farnesyl diphosphate synthase; GGPPS, geranylgeranyl diphosphate synthase; DTT, dithiothreitol; Mopso, 3-(N-morpholino)-2-hydroxypropane-sulfonic acid.
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preparation and immunoblotting protocols were described earlier (14), as were radio-gas chromatography methods (21).

Generation of Plasmid Constructs—A truncated version of the GPP synthase small subunit (designated GPPS.ssu), in which the plastidial transit peptide was deleted, was prepared from the original pS-BET13.18 cDNA clone (14) for transfer into pET-37b using forward primer (5′-CC GCC GCC CAT ATG CAG CCG-3′) to create an NdeI site and code for a starting methionine (in place of amino acid Ser-48 of the original protein) and reverse primer (5′-G GAT AGG CAG AAT CTT CTA AGC CGG-3′) to generate a HindIII restriction site downstream of the stop codon. The resulting amplicon was digested with NdeI and HindIII, gel-purified, and directionally ligated into the NdeI/HindIII-digested pET-37b vector to yield pETGPPS.ssu. Mutation of the stop codon, to permit translation through the carboxyl-terminal His6-tag provided on the pET-37b vector, was accomplished by PCR amplification using pETGPPS.ssu as template and forward primer (5′-GGA GAT ATC CAT ATG CAG CCG-3′) and reverse primer (5′-CGC AGG AAT CTT CCC AGC CGG-3′), thereby resulting in a lysine substitution for the stop codon six residues from the His6-tag. The resulting amplicon (designated GPPS.ssu.his) was digested and gel-purified as before and directionally ligated into pET-37b that had been digested with NdeI and HindIII.

A similarly truncated version of the GPP synthase large subunit was prepared from the original pMP23.10 cDNA clone (14) using forward primer (5′-CTA GCG CCC CTG TAC TCT TCA TCT TCA GCC AGG-3′) to convert the downstream KpnI site (GTTACC) in the original pluScript vector (14) (GGATCC). The amplified sequence (designated GPPS.slu) was digested with NdeI and BamHI, and the gel-purified fragment was ligated into pET-32a that had been similarly digested to yield pETGPPS.slu. A truncated version of the Taxis canadensis GGPP synthase, from which the amino-terminal plastid targeting peptide was also deleted, was prepared from the original full-length cDNA clone (22) using forward primer (5′-CCG CAA AGA CAT ATG TTT GAT TTC AGT GGA-3′, to install an NdeI restriction site and thus mutate Glu-98 to Met-1, and reverse primer (5′-CTG AGC AGA CAT ATG TCT TCT CTG-3′) to create the downstream KpnI site). A truncated version of the Abies grandis GGPP synthase was similarly prepared.

An FPP synthase cDNA clone was prepared from a full-length sequence located in a peppermint (M. piperita) oil gland expressed sequence tag library (23) using primer (5′-GGG TGA TTA CAT ATG GCG AAT C-3′) to create an NdeI restriction site at the starting methionine and reverse primer (5′-GAT AGT CAC TCC GAT GCT GTA TTT CAC-3′) to create a XhoI restriction site downstream of the stop codon. The resulting amplicon was digested with NdeI and XhoI, gel-purified, and ligated into the pET-32c vector that had been identically digested to yield pETGPPS.slu. A truncated version of the Abies grandis GGPP synthase was similarly prepared.

Transformations and Expression in E. coli—Cotransformations with two plasmids (e.g. both GPP synthase subunits and pETGPPS.ssu.his with pETTPPS or pETGGPPS) were performed in a single transformation event. Positive transformants were screened for multiple resistance with kanamycin (pETGPPS.slu and pETGPPS.ssu.his), carbenicillin (pETGPPS.slu, pETGGPPS, and pETTPPS), and chloramphenicol (conferring the pACYC-based plasmid of the BL21-Codon Plus host cells that also contains extra copies of the argU, ileY, and leuW tRNA genes). Transformants were initially grown in 5 ml of Luria-Bertani medium and then transferred to 1 liter of the same medium and grown at 20 °C until A600 reached 0.6. The temperature of the culture was then lowered to 15 °C prior to induction with 0.4 mM isopropyl-1-thio-β-D-galactopyranoside, and incubation was continued for an additional 16 h.

Protein Purification—Following induction and incubation, the transformed cells were pelleted (20 min at 5000 × g) and resuspended in either 20 ml of buffer A or buffer B (see buffer description below) or in 10 ml of His-tagged lysis buffer (50 mM NaH2PO4 (pH 8.0), 300 mM NaCl, 1 mM dithiothreitol (DTT), 1 mM benzamidine, and 10 mM histidine). The suspended cells were disrupted by brief sonication (VirSonic, 300 W, 10 s; four 30-s bursts at 4°C) and then homogenized in a French press at 12,000 × g (30 min) to pellet debris and then at 195,000 × g (1.5 h) to provide the soluble enzyme fraction that was filtered through a cellulose acetate membrane (Nalgene, 0.2 μm).

Crude recombinant GPP synthase was purified by fast protein liquid chromatography (Amerham Biosciences) using an HR 10/10 column (Amerham Biosciences) containing Poros PI anion-exchange medium
Identical residues (two or more) are shown in black (GGPPS), the GPP synthase large subunit from *canadensis*.

2 M NaCl and applied to an HR 10/10 column containing phenyl-

mM MgCl2 was loaded into a 12-mm double-sector cell containing 3-mm confidence level, determined using NONLIN (25)) and a partial specific volume (0.7427 ml/g determined from amino acid sequence) calculated in SEDNTERP (24).

Prenyltransferase Assays—Assays used to determine steady-state kinetic constants and to evaluate product chain length distribution were based on the acid-lability procedure (26, 27) in which the product allylic diposphates are solvolyzed to mixtures of the corresponding cis, trans, and tertiary allylic alcohols that are partitioned from the remaining unincorporated (acid-stable) IPP by hexane extraction. For kinetic assays, 50–200 nmol of enzyme was combined with 100 mM Mopso buffer (pH 6.7) containing the appropriate amounts of MgCl2, [4–31P]IPP, and the allylic cosubstrate (DMAPP, GPP, or FPP) in a total volume of 100 µl. The mixtures were incubated for 2 min, and the linear reaction was then quenched with 100 µl of a 9:1 (v/v) methanol:3 НCl solution. The acidified mixture was then overlayed with 1 ml of hexane, shaken for 10 min to allow completion of solvolyis, and then centrifuged briefly to separate the aqueous layer from the hexane layer (containing allylic alcohols), an aliquot of which was measured by liquid scintillation counting. Kinetic data were evaluated using Enzyme Kinetics software (Trinity Software) and the Hanes-Woolf algorithms. Reported values for substrate $K_M$ are the means of three experiments; the reported $K_M$ values for MgCl2 are the means of two experiments.

Preparative assays used for product analysis were similar to those described above with the exceptions that 400 ng of protein was employed at saturating substrate concentrations, and the reaction time was extended to 10 min followed by the addition of 1 ml of 100 mM Tris buffer (pH 9.5) containing 10 units each of bovine alkaline phosphatase and potato apyrase to hydrolyze the diphosphate esters. The reaction mixture was overlaid with pentane and allowed to incubate for 3 h at 31 °C, after which the contents were vigorously mixed and centrifuged to separate phases. The extraction procedure was repeated twice with 1-ml portions of diethyl ether, and the combined organic extract was dried over Na2SO4, diluted with internal standards, and concentrated under N2 (to 20 µl) for radio-gas chromatographic analysis.

**RESULTS**

Expression, Purification, and Characterization of GPP Synthase—Unlike other short-chain prenyltransferases that are homodimers (1), GPP synthase is composed of two subunits, a large subunit (GGPPS.lsu) that resembles GGPPS (but when expressed alone is inactive in prenyltransfer catalysis) and a small, similarly inactive, subunit (GPPS.ssu) that does not closely resemble any known prenyltransferase but appears to...
confer function and chain length specificity in the subunit interaction (14). To investigate whether GPP.ssu could interact with other prenyltransferases (FPPS and GGPPS) to influence chain length distribution of the prenyl diphosphate products, it was first necessary to develop suitable expression systems and purification methods for characterizing the target proteins. Heterologous expression in E. coli of functional GPPS had been demonstrated previously (14), yet the amount of soluble recombinant protein produced was low due to the propensity to form inclusion bodies.

Both GPPS (28, 29) and GGPPS (30, 31) of plant origin have been localized to plastids. Thus, these nuclear gene products are expected to be translated as preproteins bearing an amino-terminal plastidial transit peptide for directing organellar import and subsequent proteolytic processing to the mature form (32, 33). Both GPP.ssu and GPP.slsu appear to be translated as such preproteins based on the comparison of the deduced amino-terminal sequences (14) to those of other transit peptides (34). Previous studies with other plastidial enzymes, including monoterpene and diterpene synthases (35, 36) and GGPPS (22), have demonstrated higher levels of heterologous expression of soluble, more readily purified proteins when truncated to resemble the mature forms. Therefore, both the GPPS small and large subunits were truncated for expression from the pET vector. The truncation sites selected (Fig. 2) yielded proteins of a size consistent with the molecular weights of the native subunits previously determined by SDS-PAGE (14).

Coexpression in E. coli of the truncated versions (pETGFP.ssu and pETGFP.slsu) demonstrated substantially improved expression over the previously employed preprotein forms and a higher net yield of recombinant enzyme when both subunits were expressed simultaneously (data not shown). Purification of the recombinant GPPS (from coexpressed pETGFP.ssu and pETGFP.slsu) by the procedure described yielded greater than 5 mg of protein (>90% purity) per liter of culture, as judged by SDS-PAGE (Fig. 3). Polyclonal antibodies raised against the mature subunit versions of GPPS (14) readily detected the corresponding truncated subunit versions of the enzyme in these preparations, at a ratio of 1:1 (Fig. 4, B and C). Radio-gas chromatographic analysis of the enzymatically dephosphorylated reaction products confirmed that the recombinant, truncated GPPS produced exclusively geranyl diphosphate (Fig. 5B).

Gel permeation chromatography indicated that the size of GPP synthase was 135 ± 5 kDa (Fig. 6A), thus suggesting a tetrameric protein. To confirm this result, the recombinant GPPS was also evaluated by analytical ultracentrifugation, from which a deduced size of 134 ± 5 kDa was obtained (data not shown). These measurements, combined with the results of SDS-PAGE, which show equal amounts of the two subunits, indicate that recombinant GPP synthase is a tetramer composed of two small subunits (of 27 kDa each) and two large subunits (of 35 kDa each); the empirically determined molecular weight is within 9% of the calculated value. Because previous analysis of the recombinant, preprotein form of GPPS had indicated a size of 68 ± 6 kDa (14), the native version of GPPS from peppermint oil glands was also analyzed. By activity assay, GPP synthase was observed in this instance to elute in column fractions corresponding to sizes of 140 (~70% of total activity) and 66 kDa (~30% of total activity) (Fig. 6B), and both molecular weight forms produced exclusively GPP as product (in proportion to the amount of synthase protein) as determined by radio-gas chromatographic analysis (data not shown). These results demonstrate that heterodimeric and heterotetrameric (a dimer of the heterodimer) forms of GPPS exist naturally and that both are catalytically active; the concentration dependence of the distribution of dimer and tetramer, which may underlie the earlier observation of only the dimeric form (14), is being examined. It is interesting to note that GPP.ssu, when expressed alone, forms homodimers of 54 kDa as determined by gel permeation (Fig. 6A); the large subunit, when expressed alone, apparently does not self-associate (data not shown).

Kinetic evaluation of the recombinant, truncated version of GPPS (tetrameric form) provided an apparent $K_M$ (DMAPP) value of 54 μM and a $K_M$ (MgCl₂) value of 2.1 mM, which are within the range of values previously reported for partially purified, native GPP synthases from other sources; however, the apparent $K_M$ (IPP) value of 26 μM is 2–4 times higher than values reported for other prenyltransferases of this type (Table 1 (21, 37–39)). GPPS was unable to utilize GPP or FPP as the allylic cosubstrate with IPP, consistent with the observed chain length specificity of this enzyme. A $k_{cat}$ value of 4.8 s⁻¹ was determined for GPPS. This appears to be the first reported turnover rate for any GPPS and is 10-fold higher than the rate reported for the well characterized avian FPPS (9).

Expression, Purification, and Characterization of GGPP Synthase—A cDNA encoding GGPP synthase from Canadian yew
single protein band indicated by SDS-PAGE (at 32 kDa), combined with an estimated size of 66 kDa by gel permeation chromatography (Fig. 6A), confirms that this GGPP synthase is a functional homodimer. Polyclonal antibodies raised against the *Mentha* GPPS large subunit detected the *Taxus* GGPPS (Fig. 4C). Radio-gas chromatographic analysis demonstrated that geranylglycerol diphosphate was the exclusive product of this enzyme when DMAPP and IPP were employed as cosubstrates (Fig. 5E; note that geranyllinalool eluting at 30–40 min is an artifact of the rearrangement of geranylglycerol during the analysis as observed previously (22, 40)).

Kinetic evaluation of the purified *Taxus* GGPPS (Table I) confirmed that this enzyme accepts DMAPP ($K_M$ 127 μM; $k_{cat}$ 1.0 s$^{-1}$), GPP ($K_M$ 7.7 μM; $k_{cat}$ 2.1 s$^{-1}$), or FPP ($K_M$ 2.6 μM; $k_{cat}$ 3.3 s$^{-1}$) as the allylic cosubstrate. Michaelis constants reported here are in the range of values described previously for other GPP synthases (1, 40–42); turnover rates for other GPP synthases have not been reported. It is notable that $k_{cat}$ increases in direct proportion to the size of the allylic cosubstrate (i.e. the rate of formation of GGPP from DMAPP (involving three condensation steps) is one-third that from FPP (involving a single condensation step)). The $K_M$ values for GPP and FPP are also substantially lower than that of DMAPP, consistent with the inability to detect either GPP or FPP as free intermediates in the elongation sequence when DMAPP is used as the cosubstrate.

**Expression, Purification, and Characterization of FPP Synthase**—A cDNA encoding a full-length FPP synthase was previously identified in a peppermint (*M. piperita*) oil gland-expressed sequence tag project (23). Unlike GPP synthase and GGPP synthase, this FPP synthase is devoid of an organellar targeting sequence and, consequently, is cytosolic. Expression from the pET vector in *E. coli*, and purification of the recombinant protein, yielded 20 mg/liter FPP synthase at greater than 90% purity by SDS-PAGE (Fig. 4A); radio-gas chromatographic analysis confirmed that FPP was the predominant product (Fig. 5D). The enzyme was similar in properties to other FPP synthases of plant origin (21).

**Expression, Purification, and Characterization of Hybrid Prenyltransferases**—To determine whether the GGPPS small subunit could interact with FPP synthase or GGPP synthase to alter chain length distribution of the product, a purification procedure was developed that could separate heteromultimeric forms containing the GPPS.ssu from recombinant FPP synthase and GGPP synthase homodimers. A histidine tag (His$_6$-tag) was appended to the carboxyl terminus of GPPS.ssu (designated GPPS.ssu.his) to permit separation of heteromeric species (GPPS.ssu.his/GGPPS and GPPS.ssu.his/FPPS) from the homodimeric GGPPS and FPPS by metal ion affinity chromatography. Similar histidine tagging has been used successfully in the purification of human GGPP synthase (43). To test the binding efficiency for GPPS.ssu.his on the affinity matrix and to ensure that the carboxyl-terminal tag did not interfere with the function of GPPS.ssu, GPPS.ssu.his and GPPS.ssu.were coexpressed (the resulting protein is designated GPPS.his), and the derived soluble protein fraction was applied to the affinity column. Following washing, the bound material was eluted with 250 mM histidine and was shown by SDS-PAGE to consist of two proteins in equal amounts and of sizes expected for the GPP synthase subunits (Fig. 4A). Immunoblotting demonstrated that rabbit anti-GPPS.ssu and anti-GPPS.ssu recognized the His$_6$-tagged protein version and confirmed that both large and small subunits were present in the complex (Fig. 4, B and C). These results indicated that any heteromeric subunit assemblies containing the His$_6$-tagged small subunit could be purified by immobilized Ni$^{2+}$-affinity chromatography. Gel

(T. canadensis) was previously isolated and functionally expressed in yeast (22). This prenyltransferase was chosen to evaluate the possible interaction with the GGPPS small subunit because *Taxus* is phylogenetically distant from *Mentha*, and the *Taxus* GGPP synthase bears the lowest level of deduced sequence identity to the GGPPS large subunit than does any other functionally documented GGPPS of plant origin (57% at the amino acid level; see Fig. 2), thereby providing a challenging and most useful test of small subunit function.

Both the preprotein and truncated versions of GGPPS (Fig. 2) were expressed in *E. coli* from the pET vector, and protein production was monitored by SDS-PAGE. Expression of the preprotein yielded predominantly inclusion bodies, whereas the truncated cDNA provided mostly soluble protein (Fig. 3B). Purification of the truncated form of GGPPS provided up to 10 mg of protein (at >90% purity) per liter of culture (Fig. 4A).
permeation chromatography (Fig. 6A) demonstrated that the GPPS.his species had a size of 140 ± 10 kDa, thereby indicating that the appended His₈-tag did not interfere with tetramer assembly. Kinetic evaluation (Table I) and product analysis (Fig. 5C) demonstrated that GPPS.his was comparable in catalytic properties to the original recombinant GPPS and that the carboxyl-terminal tag did not compromise GPPS.ssu function.

To determine whether GPPS.ssu.his could interact productively with the Taxus GGPP synthase, the corresponding constructs (pETGGPPS and pETGPPS.ssu.his) were coexpressed in E. coli, and the resulting soluble recombinant protein was purified by affinity chromatography as before. The similarly purified enzyme preparation from E. coli transformed with pETGGPPS alone was employed as the control. In both cases, the flow-through and wash fractions from the affinity column

![Fig. 6](image)

**Fig. 6.** Size determination of GPP synthase by gel permeation chromatography and SDS-PAGE. The elution behavior is illustrated for purified, recombinant GPPS (A) and partially purified, native GPPS (B) by gel permeation chromatography on Superdex 200. The top panel in A shows the absorbance profile (A₂₈₀) and assayed activity profile (dpm) for recombinant GPPS. B shows the corresponding profiles for the native enzyme from peppermint oil glands. A, the middle panel depicts the elution behavior of the standards amylase (200 kDa), alcohol dehydrogenase (150 kDa), and bovine serum albumin (66 kDa), and carbonic anhydrase (29 kDa). The lower panel illustrates the elution behavior of the various prenyltransferases on the same sizing column by SDS-PAGE analysis of the corresponding (V/V₀) column fractions.

**TABLE I**

**Apparent KM values and rate constants for recombinant prenyltransferases**

The abbreviations used in this table are as follows: GPPS, geranyl diphosphate synthase (truncated versions of both subunits); GPPS.his, geranyl diphosphate synthase (truncated versions of both subunits with the His-tagged small subunit); GGPPS, geranylgeranyl diphosphate synthase (truncated version); GPPS.ssu.his/GGPPS, chimeric prenyltransferase composed of truncated version of GGPPS and truncated, His-tagged version of GPPS small subunit.

| Enzyme               | DMAPP (+IPP)  | GPP (+IPP)  | FPP (+IPP)  | IPP (+DMAPP) | IPP (+GPP)  | IPP (+FPP)  | MgCl₂ |
|----------------------|---------------|-------------|-------------|--------------|-------------|-------------|-------|
|                      | KM (µM)       | kcat (µM⁻¹ s⁻¹) | KM (µM)     | kcat (µM⁻¹ s⁻¹) | KM (µM)     | kcat (µM⁻¹ s⁻¹) |       |
| GPPS                 | 54 ± 3        | 39 ± 2      | 202 ± 3     | 50 ± 1       | 26 ± 3      | 18 ± 2      | 12  |
| GPPS.his             | 63 ± 8        | 66 ± 2      | 205 ± 3     | 50 ± 2       | 41 ± 2      | 22 ± 1      | 34  |
| GGPPS/GPPS.ssu.his   | 75 ± 3        | 48 ± 2      | 215 ± 3     | 50 ± 2       | 42 ± 2      | 23 ± 1      | 36  |
| GPPS.ssu             | 127 ± 10      | 50 ± 2      | 263 ± 3     | 50 ± 2       | 45 ± 2      | 25 ± 1      | 38  |

**a** At saturating concentrations of IPP, 300 µM with DMAPP and GPP, and 400 µM with FPP.

**b** At saturating concentrations of the allylic cosubstrate, 300 µM for DMAPP, 230 µM for GPP, and 100 µM for FPP.

**c** At saturating concentrations of IPP and allylic cosubstrate.

**d** At saturating concentrations of IPP and DMAPP.

**e** ND, activity not detected.
were shown by SDS-PAGE to contain a prominent 32-kDa band (Fig. 7, C and D) that was shown by immunoblotting with anti-GPPS.ssu.his to be the Taxus GGPPS (data not shown). Affinity elution with 250 mM histidine, in the case of the coexpression system, revealed the presence of equal amounts of prominent proteins at 32 and 27 kDa that were entirely absent in the control and that were identified by immunoblotting as GGPPS and GPPS.ssu.his (Fig. 4, B and C). This heteromeric protein was subsequently purified by strong anion-exchange chromatography, through which the associated GGPPS and GPPS.ssu.his remains intact (Fig. 7D). Gel permeation chromatography of the associated species provided a size of 62 kDa (Fig. 6A), indicating that, unlike GPPS, the GGPPS/GPPS.ssu.his is a heterodimer. Radio-gas chromatographic analysis demonstrated that the GGPPS/GPPS.ssu.his heterodimer produced predominantly geranyl diphosphate as product, with trace amounts of farnesyl diphosphate and geranylgeranyl diphosphate, when supplied with DMAPP as the allylic cosubstrate (Fig. 5F); however, unlike GPPS, the GGPPS/GPPS.ssu.his hybrid dimer also accepts GPP and FPP as allylic substrates to yield, respectively, FPP with lesser amounts of GGPP (Fig. 5G), and exclusively GGPP (Fig. 5H), as products.

Kinetic evaluation of the hybrid dimer (GGPPS/GPPS.ssu.his; Table I) indicated that the apparent $K_M$ values for DMAPP and for IPP (with DMAPP as cosubstrate) were very similar to those of GPP synthase, and that $K_M$ values for GPP and FPP were remarkably similar to those of GGPP synthase. With GPP or FPP as cosubstrates, however, the $K_M$ values for IPP with the hybrid dimer differed from those of GGPP synthase (one lower and one higher; Table I). The overall rate of prenyltransferase activity was highest for the hybrid dimer when supplied with DMAPP as cosubstrate (i.e., for GPP synthesis) and thus opposite to that observed for the native GGPPS (Table I).

To probe further this unusual phenomenon, another phylogenetically distant GGPPS was tested. In this case, the cDNA (GenBank™ accession no. AF425235; 56% identical to the GGPPS large subunit) encoding GGPPS from grand fir (A. grandis) (21) was first expressed alone, as before, purified to homogeneity, and confirmed to produce essentially only GPP (trace level of GPP) with IPP and DMAPP as cosubstrates. The Abies GGPPS was then coexpressed with pETGGPS.ssu.his, and the resulting soluble recombinant protein was purified by affinity chromatography. Analysis of the protein eluted with 250 mM histidine, by SDS-PAGE as before, revealed the presence of equal amounts of the Abies GGPPS and GPPS.ssu.his, thus indicating an equimolar association as observed previously with the Taxus GGPPS and the Mentha GPPS small subunit.

Assay of this heteromeric enzyme with IPP and DMAPP yielded essentially only GPP as product (trace of FPP), and at a rate comparable to the formation of the C_{20} product by the parent GGPPS homodimer. This second example confirmed the ability of the GPPS small subunit to modify the specificity of GGPPS for production of the shorter prenyl chain.

To determine whether GPPS.ssu.his could associate with and catalytically influence FPP synthase, an experiment of identical format to that described above was performed, in which pETGPPS.ssu.his and pETFPPS were coexpressed in E. coli, and the resulting recombinant protein was separated by affinity chromatography. In this case, the purified enzyme preparation from E. coli transformed with pETFPPS alone served as the control. SDS-PAGE analyses of both preparations showed that the bulk of the FPPS (at 45 kDa) eluted in the flow-through and wash fractions from the affinity column (Fig. 7, A and B). Elution of the affinity column with 250 mM histidine yielded only traces of the recombinant FPPS (at 45 kDa) in both cases, and the bulk of the GPPS.ssu.his (at 27 kDa) in the case of the coexpression experiment; this protein, as anticipated, was absent in the control experiment in which FPPS was expressed alone (Fig. 7, A and B). Assay of each protein fraction with DMAPP plus IPP showed by radio-gas chromatographic analysis that the only product formed in all instances was FPP (data not shown). These data indicated that, although GPPS.ssu.his and FPPS were successfully coexpressed, no interaction between the two occurred that was sufficient to permit isolation of an associated species or to promote a detectable alteration in product formation by FPPS.

**DISCUSSION**

The observation that Mentha geranyl diphosphate synthase is composed of a large subunit that resembles geranylgeranyl diphosphate synthase and a small subunit that does not resemble any known prenyltransferase suggested a role for the small subunit in interacting with the prenyltransferase-like large subunit to promote catalysis while restricting the reaction to a single condensation cycle (with DMAPP and IPP) to produce specifically geranyl diphosphate. To determine whether the small subunit could interact with other prenyltransferases to influence product distribution, a strategy was developed that employed a histidine-tagged version of the GPPS small subunit to permit affinity-based purification of this subunit along with any associated protein. This approach was tested with the GGPPS from Taxus which bears the least resemblance of any extant GGPPS to the Mentha GPPS large subunit. Following coexpression, the resulting chimeric prenyltransferase was purified and shown to synthesize primarily GPP with competent kinetics. These results, which were confirmed with the GGPPSs from A. grandis, clearly demonstrate that the small subunit of GPPS is capable of binding to and modifying GGPPSs to promote the efficient production of GPP by compromising the subsequent elongation steps to FPP and GGPP. The physical basis of subunit interactions that underlies this catalytic alteration is unknown and is presently under investigation. Because the small subunit lacks the aspartate-rich motifs associated
with prenyltransferase chemistry, a direct role in catalysis seems unlikely but rather suggests that the small subunit serves to bind to and constrict the GGPPS active site cavity, thereby restricting the ability to conduct elongation beyond the C10 stage. Comparing hydrophathy plots for GPPS.ssu, there is no specific requirement for GPP production at this stage of transit of the small subunit from cytosol to plastid. Therefore, there is no functional utility for the interaction with another protein, i.e., the large subunit that resembles a prenyltransferase, including the kinetic competence and subunit architecture are unknown; the operation of a monoterpene-based signaling system as a response to pathogens or herbivore attack, as found in other plants (51, 52). Further studies with essential oil-producing plants and other (non-producing) species should allow determination of the significance of these two fundamentally different types of diphosphate synthases.

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Interaction with the Small Subunit of Geranyl Diphosphate Synthase Modifies the Chain Length Specificity of Geranylgeranyl Diphosphate Synthase to Produce Geranyl Diphosphate

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