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Structural Characterization of Early Michaelis Complexes in the Reaction Catalyzed by (+)-Limonene Synthase from *Citrus sinensis* Using Fluorinated Substrate Analogues

Ramasamy P. Kumar[†], Benjamin R. Morehouse[†], Jason O. Matos[†], Karan Malik[†], Hongkun Lin[†], Isaac J. Krauss[‡], and Daniel D. Oprian^{*,†}

[†]Department of Biochemistry, Brandeis University, Waltham, Massachusetts 02454, United States

[‡]Department of Chemistry, Brandeis University, Waltham, Massachusetts 02454, United States

Abstract

The stereochemical course of monoterpene synthase reactions is thought to be determined early in the reaction sequence by selective binding of distinct conformations of the geranyl diphosphate (GPP) substrate. We explore here formation of early Michaelis complexes of the (+)-limonene synthase [(+)-LS] from *Citrus sinensis* using monofluorinated substrate analogues 2-fluoro-GPP (FGPP) and 2-fluoroneryl diphosphate (FNPP). Both are competitive inhibitors for (+)-LS with K_i values of 2.4 ± 0.5 and $39.5 \pm 5.2 \mu\text{M}$, respectively. The K_i values are similar to the K_M for the respective nonfluorinated substrates, indicating that fluorine does not significantly perturb binding of the ligand to the enzyme. FGPP and FNPP are also substrates, but with dramatically reduced rates (k_{cat} values of 0.00054 ± 0.00005 and $0.00024 \pm 0.00002 \text{ s}^{-1}$, respectively). These data are consistent with a stepwise mechanism for (+)-LS involving ionization of the allylic GPP substrate to generate a resonance-stabilized carbenium ion in the rate-limiting step. Crystals of apo-(+)-LS were soaked with FGPP and FNPP to obtain X-ray structures at 2.4 and 2.2 Å resolution, respectively. The fluorinated analogues are found anchored in the active site through extensive interactions involving the diphosphate, three metal ions, and three active-site Asp residues. Electron density for the carbon chains extends deep into a hydrophobic pocket, while the enzyme remains mostly in the open conformation observed for the apoprotein. While FNPP was found in

***Corresponding Author:** Department of Biochemistry, Brandeis University, 415 South St., Waltham, MA 02454. Telephone: 781-736-2322. Fax: 781-736-8487. oprian@brandeis.edu.

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.bio-chem.7b00144. Gas chromatogram and circular dichroism spectrum for the product of NPP, metal ion coordination geometry, and overall superposition of apo- and FGPP-(+)-LS (PDF)

Accession Codes

The atomic coordinates and structure factors have been deposited in the Protein Data Bank as entries 5UV1 and 5UV2 for FGPP- and FNPP-bound (+)-limonene synthase, respectively.

ORCID

Benjamin R. Morehouse: 0000-0003-3352-5463

Daniel D. Oprian: 0000-0002-6520-5459

Author Contributions

R.P.K. and B.R.M. contributed equally to this work.

Notes

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multiple conformations, FGPP, importantly, was in a single, relatively well-defined, left-handed screw conformation, consistent with predictions for the mechanism of stereoselectivity in the monoterpene synthases.

Graphical abstract

Terpene synthases catalyze the committed step in the biosynthesis of the chemically diverse family of terpenoid natural products.^{1,2} Using simple diphosphorylated prenyl precursors as substrates, these enzymes catalyze the formation of terpenes by generating and controlling the reactivity of high-energy carbenium ion intermediates. The divalent metal ion-independent reactions can involve ring formation, carbon skeleton rearrangements, and methyl and hydride shifts and are characterized by high stereoselectivity. In-depth elucidation of terpene synthase mechanisms is a prerequisite for harnessing the remarkable activities of these enzymes.

Limonene synthase catalyzes the simplest of the terpene cyclization reactions, transforming the C₁₀ precursor geranyl diphosphate (GPP) into the volatile monocyclic terpene limonene.² Limonene is found in nature as two different enantiomers, (*R*) and (*S*) [or (+) and (–), respectively]. (–)-Limonene synthase [(–)-LS] has been developed as a model system for understanding monoterpene biosynthesis through the early pioneering studies of Croteau and coworkers.^{3–5} (+)-LS is an attractive complementary model system, especially for investigation of factors that contribute to stereochemical control of reactions involving the high-energy carbenium ion intermediates.

While the reaction mechanism for (+)-LS from *Citrus sinensis* has not previously been explored in extensive detail, related studies with (–)-LS from spearmint and other monoterpene synthases suggest the following scenario (Figure 1).² Cyclization is thought to begin with stereoselective binding of GPP in a left-handed screw conformation.^{6,7} Ionization of the allylic diphosphate to generate the resonance-stabilized allylic carbenium ion (a step thought to be rate-limiting for enzymatic turnover) is followed by *syn* migration⁸ of

pyrophosphate to C3 to give the (4*R*)-linalyl diphosphate (LPP) intermediate.^{2,6,7} Rotation about the resulting C2–C3 bond places C1 in position for electrophilic attack on C6 from an *anti-endo* conformation. Ionization of the allylic diphosphate followed by *anti*-S_N1' cyclization produces the (4*R*)- α -terpinyl cation with net retention of configuration at C1.⁹ Finally, the reaction terminates with deprotonation of the *cis*-methyl group (*cis* in the GPP substrate) to generate the (4*R*)-limonene product.¹⁰

In the preceding paper in this issue, we describe the isolation of cDNA for a (+)-LS from the flavedo of the sweet navel orange (*C. sinensis*) (DOI: 10.1021/acs.biochem.7b00143). The gene was expressed at a high level in *Escherichia coli* to produce a pseudomature form of the enzyme truncated at the N-terminus to remove a plastidial targeting sequence. The His-tagged protein was purified to homogeneity using Ni affinity chromatography and characterized with respect to kinetics, divalent metal ion dependency, and reaction stereospecificity. The protein was also crystallized in the apo form and the X-ray structure determined to 2.3 Å resolution, permitting a comparison of structural changes linking the open conformation of (+)-LS to the closed conformation observed for (–)-LS from spearmint (*Mentha spicata*) as reported by Hyatt et al.¹¹

Here we present X-ray crystal structures of early Michaelis complex intermediates in the formation of (+)-limonene obtained by soaking crystals of the apoprotein with the substrate analogues 2-fluorogeranyl diphosphate (FGPP) and 2-fluoroneryl diphosphate (FNPP) (Figure 2). Structures for the FGPP and FNPP derivatives were determined at 2.4 and 2.2 Å resolution, respectively, and show the substrate analogues anchored in the active site through extensive interactions involving the diphosphate moiety, three metal ions, and three active-site Asp residues. Each metal ion is hexacoordinate with well-defined octahedral coordination geometry, and electron density for the carbon chain of the analogues is clearly visible, extending into a hydrophobic pocket of the active site. The protein remains mostly in the open conformation observed for the apoprotein, representative of early Michaelis complex intermediates in which a substrate has bound but the protein has yet to fully close around the ligand. While the electron density for FNPP was consistent with more than one conformation in the active site of the protein, the FGPP ligand, importantly, was found to be in a single, relatively well-defined, left-handed screw conformation just as predicted for the stereoselectivity of this reaction.^{6,7}

EXPERIMENTAL PROCEDURES

Synthesis of Neryl Diphosphate

Geranyl diphosphate (GPP) and neryl diphosphate (NPP) were synthesized from geraniol and nerol, respectively, using the large-scale phosphorylation procedure previously described by Keller and Thompson.¹² Each allylic alcohol (300 mg) was phosphorylated by reaction with triethylammonium phosphate (TEAP) and trichloroacetonitrile at 37 °C. The reaction mixture was stored overnight at –20 °C and later separated by flash chromatography on a silica column using a 12:5:1 isopropanol/ ammonium hydroxide/water mobile phase. Fractions were analyzed by thin-layer chromatography (TLC) developed in a 6:3:1 isopropanol/ammonium hydroxide/water mixture and visualized with KMnO₄.

Fractions containing the diphosphate were pooled, concentrated by rotary evaporation under reduced pressure, flash-frozen in liquid nitrogen, and lyophilized to dryness for 18–24 h.

Lyophilized NPP was further purified by anion exchange chromatography using a 1 cm × 8 cm column of DOWEX-1X2-400 strongly basic anion exchange resin, chloride form (Sigma), equilibrated with 125 mM ammonium bicarbonate (pH 8); 10–30 mg of NPP was loaded onto the column, followed by 40 mL of 125 mM ammonium bicarbonate at a flow rate of 2 mL/min before elution of NPP with 500 mM ammonium bicarbonate. Fractions were analyzed by TLC as described above, and those containing NPP were pooled, flash-frozen in liquid nitrogen, and lyophilized to dryness for 18–24 h. The lyophilized product was stored at –20 °C until it was needed. The purity of NPP (and GPP) was assessed by proton, carbon, and phosphorus nuclear magnetic resonance (NMR) spectroscopy.

All NMR spectra were recorded on a Varian 400-MR spectrometer (9.4 T, 400 MHz) in D₂O adjusted to pH ~8.0 with ND₄OD. ¹H and ¹³C chemical shifts are reported in parts per million downfield from TMS (trimethylsilylpropionic acid), and ³¹P chemical shifts are reported in parts per million relative to 85% *o*-phosphoric acid. ¹⁹F chemical shifts are reported in reference to NaF in D₂O. *J* coupling constants are reported in units of frequency (hertz) with multiplicities listed as s (singlet), d (doublet), dd (doublet of doublets), t (triplet), m (multiplet), br (broad), and app (apparent).

GPP: ¹H NMR (400 MHz, D₂O/ND₄OD) δ_{H} 1.64 (3 H, s, CH₃), 1.70 (3 H, s, CH₃), 1.73 (3 H, s, CH₃), 2.08–2.20 (4 H, m, H at C4 and C5), 4.48 (2 H, app t, *J* = 6.6 Hz, *J*_{H,P} = 6.6 Hz, H at C1), 5.22 (1 H, br t, *J* = 6.0 Hz, H at C6), 5.47 (1 H, t, *J* = 7.0 Hz, H at C2); ¹³C{¹H} NMR (100 MHz, D₂O/ND₄OD) δ_{C} 18.45, 19.81, 27.67, 28.45, 41.64, 65.32 (1 C, d, *J*_{C,P} = 5.3 Hz), 122.90 (1 C, d, *J*_{C,P} = 8.4 Hz), 127.03, 136.57, 145.43; ³¹P{¹H} NMR (162 MHz, D₂O/ND₄OD) δ_{P} –5.74 (1 P, d, *J*_{P,P} = 22.1 Hz, P1), –9.55 (1 P, d, *J*_{P,P} = 22.1 Hz, P2).

NPP: ¹H NMR (400 MHz, D₂O/ND₄OD) δ_{H} 1.63 (3 H, s, CH₃), 1.70 (3 H, s, CH₃), 1.77 (3 H, s, CH₃), 2.10–2.21 (4 H, m, H at C4 and C5), 4.46 (2 H, app t, *J* ~ 6.9 Hz, H at C1), 5.21 (1 H, br t, *J* ~ 6.9 Hz, H at C6), 5.47 (1 H, t, *J* = 7.1 Hz, H at C2); ¹³C{¹H} NMR (100 MHz, D₂O/ND₄OD) δ_{C} 19.84, 25.45, 27.71, 28.87, 34.11, 65.15 (1 C, d, *J*_{C,P} = 5.3 Hz), 123.75 (1 C, d, *J*_{C,P} = 8.1 Hz), 126.84, 136.77, 145.54; ³¹P{¹H} NMR (162 MHz, D₂O/ND₄OD) δ_{P} –6.40 (1 P, d, *J*_{P,P} = 22.1 Hz, P1), –9.62 (1 P, d, *J*_{P,P} = 22.1 Hz, P2).

Synthesis of 2-Fluorogeranyl Diphosphate and 2-Fluoroneryl Diphosphate

The synthesis of 2-fluorogeraniol was performed as described by Miller et al.¹³ In brief, Horner–Wadsworth–Emmons reaction of 6-methyl-5-hepten-2-one with triethyl 2-fluoro-2-phosphonoacetate using NaH as a base was followed by reduction with DIBAL-H and resolution of the (*Z*) and (*E*) vinylic fluorides by flash chromatography on silica. Assignment of (*Z*) and (*E*) alcohols was initially assumed according to the elution order reported by Miller et al. but later confirmed by nuclear Overhauser effect (NOE) after conversion to pyrophosphates (*vide infra*). The allylic alcohols were converted to FGPP and FNPP by reaction with TEAP in trichloroacetonitrile as described above for the phosphorylation of the nonfluorinated substrate alcohols.

FGPP: ^1H NMR (400 MHz, $\text{D}_2\text{O}/\text{ND}_4\text{OD}$) δ_{H} 1.63 (3 H, s, CH_3), 1.70 (3 H, s, CH_3), 1.73 (3 H, d, $J_{\text{H,F}} = 2.86$ Hz, CH_3), 2.13–2.20 (4 H, m, H at C4 and C5), 4.6 (2 H, dd, $J_{\text{H,P}} = 6.0$ Hz, $J_{\text{H,F}} = 23.7$ Hz, H at C1), 5.19–5.25 (1 H, m, H at C6); ^{19}F NMR (376 MHz, $\text{D}_2\text{O}/\text{ND}_4\text{OD}$) $\delta_{\text{F}} -123.05$ (1 F, t, $J_{\text{F,H}} = 23.7$ Hz); $^{31}\text{P}\{^1\text{H}\}$ NMR (162 MHz, $\text{D}_2\text{O}/\text{ND}_4\text{OD}$) $\delta_{\text{P}} -6.14$ (1 P, d, $J_{\text{P,P}} = 22.1$ Hz, P1), -10.05 (1 P, d, $J_{\text{P,P}} = 22.1$ Hz, P2). The (*Z*) configuration of the double bond was confirmed by observation of an NOE between the C1 protons at 4.6 ppm and the allylic methyl protons at 1.73 ppm (but not C4/C5 protons).

FNPP: ^1H NMR (400 MHz, $\text{D}_2\text{O}/\text{ND}_4\text{OD}$) δ_{H} 1.62 (3 H, s, CH_3), 1.70 (3 H, s, CH_3), 1.71 (3 H, s, CH_3), 2.12–2.19 (4 H, m, H at C4 and C5), 4.58 (2 H, dd, $J_{\text{H,P}} = 6.0$ Hz, $J_{\text{H,F}} = 23.7$ Hz, H at C1), 5.15–5.22 (1 H, m, H at C6); ^{19}F NMR (376 MHz, $\text{D}_2\text{O}/\text{ND}_4\text{OD}$) $\delta_{\text{F}} -121.67$ (1 F, t, $J_{\text{F,H}} = 24.5$ Hz); $^{31}\text{P}\{^1\text{H}\}$ NMR (162 MHz, $\text{D}_2\text{O}/\text{ND}_4\text{OD}$) $\delta_{\text{P}} -5.62$ (1 P, br d, $J_{\text{P,P}} = 20.9$ Hz, P1), -10.03 (1 P, d, $J_{\text{P,P}} = 20.92$ Hz, P2). The (*E*) configuration of the double bond was confirmed by observation of an NOE between the C1 protons at 4.58 ppm and the C4 protons at 2.15 ppm (but not the methyl resonances).

Protein Expression and Purification

An N-terminally His₆-tagged and truncated (+)-LS construct from *C. sinensis* (residues 53–607) was expressed using a pET-28a (+) vector into BL21-CodonPlus(DE3)-RIL *E. coli* cells (Agilent Technologies) and purified by Ni²⁺ affinity chromatography as described in the preceding paper in this issue (DOI: 10.1021/acs.biochem.7b00143).

Enzymatic Activity and Inhibition Assays

Enzymatic activity was monitored using the discontinuous single-vial assay described previously (DOI: 10.1021/acs.biochem.7b00143 and ref¹⁴). The progress of the reactions was monitored by gas chromatography and mass spectrometry (GC–MS) of samples taken from the hexane layer. Product yields were determined by comparing integrated GC peaks from the reaction mixture to those of a standard curve for (+)-limonene obtained from a commercial source. The resulting velocity versus substrate concentration data for NPP were fit by nonlinear regression (Igor Pro software package, WaveMetrics) with the Michaelis–Menten equation [$v = (V_{\text{max}}[\text{S}])/(K_{\text{M}} + [\text{S}])$] to extract the kinetic parameters K_{M} and k_{cat} . Inhibition assays were conducted at fixed concentrations of the substrate analogue (FGPP or FNPP) in the presence of variable concentrations of GPP (5 μM to 1.5 mM). The reactions were allowed to proceed for various times (ranging from 1 to 6 min) before the mixtures were vortexed to stop the reaction and extract products to the hexane layer. Lineweaver–Burk double-reciprocal plots ($1/v$ vs $1/[\text{S}]$) were used to establish the type of inhibition being observed, and a plot of the apparent K_{M} versus inhibitor concentration was used to determine K_{I} values. All inhibition assays were performed in duplicate.

Gas Chromatography and Mass Spectrometry (GC–MS)

Hexane extractable terpene products were identified and quantified using GC–MS (Agilent Technologies 7890A GC System coupled with a 5975C VL MSD with a triple-axis detector). Pulsed-splitless injection was used to apply 5 μL samples to a HP-5ms (5%-phenyl)-methylpolysiloxane capillary GC column (Agilent Technologies, 30 m $\times$ 250 μm $\times$ 0.25 μm) at an inlet temperature of 220 $^{\circ}\text{C}$. The samples were run at constant pressure using

helium as the carrier gas. Samples were initially held at an oven temperature of 50 °C for 1 min, followed by a linear temperature gradient of 13 °C/min to 141 °C and a second linear gradient of 50 °C/min to a final temperature of 240 °C, which was then held for 1 min. Retention times coupled with mass fragmentation patterns were verified using commercially available terpene standards.

Preparation of Crystals for X-ray Analysis

Apoprotein crystals, obtained as described in the preceding paper in this issue (DOI: 10.1021/acs.biochem.7b00143), were presoaked in mother liquor containing 10 mM MnCl_2 for 1 h and then transferred to mother liquor containing 2 mM FGPP or FNPP and 10 mM MnCl_2 for 1 h before being flash-frozen in liquid nitrogen. All soaking steps were performed at room temperature in solutions containing 20% glycerol as a cryoprotectant.

Data Collection, Processing, and Refinement

Data sets were collected at beamline 8.2.1 at the Advanced Light Source (Lawrence Berkeley National Laboratory, Berkeley, CA) and processed as described in the preceding paper in this issue (DOI: 10.1021/acs.biochem.7b00143). The best crystals diffracted to 2.4 Å [FGPP-(+)-LS] and 2.2 Å [FNPP-(+)-LS] resolution. Diffraction data were processed in the $P4_12_12$ space group for both ligand-bound forms. The unit cell dimensions were as follows: $a = b = 85.8$ Å, $c = 215.9$ Å, and $\alpha = \beta = \gamma = 90^\circ$ for the FGPP-(+)-LS crystal, and $a = b = 85.5$ Å, $c = 215.4$ Å, and $\alpha = \beta = \gamma = 90^\circ$ for the FNPP-(+)-LS crystal. Complete data collection statistics are listed in Table 1.

The structures of ligand-bound (+)-LS were determined by molecular replacement with PHASER¹⁵ using the structure of the apoprotein as a search model (PDB entry 5UV0). The molecular replacement solution found one protein monomer in the asymmetric unit for both structures, as was also the case for the apoprotein. Structures were initially refined to starting R and R_{free} of 0.230 and 0.267, respectively, for the FGPP complex structure and 0.236 and 0.268, respectively, for the FNPP complex structure. Refinements and model building were performed as described in the preceding paper in this issue (DOI: 10.1021/acs.biochem.7b00143).

Difference Fourier electron density was observed for the FGPP- or FNPP-substrate analogue along with three metal ions in the active site of the protein. The FGPP and FNPP ligands, as well as three Mn^{2+} ions, were modeled using Jligand version 1.0¹⁶ from CCP4 software suite version 6.5,^{17,18} and the generated coordinates and restraints were used for further refinements. The disordered residues whose main chain electron density was not observed above a $1\sigma 2F_o - F_c$ cutoff were removed from the final models. Water molecules were modeled in both structures as described in the preceding paper in this issue (DOI: 10.1021/acs.biochem.7b00143). The final structures were refined to R and R_{free} values of 0.203 and 0.236, respectively, for the FGPP complex structure and 0.193 and 0.230, respectively, for the FNPP complex structure. The refinement statistics are listed in Table 1. Coordinates and structure factors for the FGPP complex (PDB entry 5UV1) and FNPP complex (PDB entry 5UV2) data sets have been deposited in the Protein Data Bank. All the crystal structure

figures in this paper were prepared using PyMol version 1.8 (Schrödinger LLC, Portland, OR).

RESULTS

Neryl Diphosphate (NPP) as a Substrate for (+)-LS

NPP, the *cis* isomer of GPP, has been shown to be a suitable alternative substrate for many monoterpene synthases, albeit typically a substrate less productive than GPP.^{4,19,20} In the case of (+)-LS, NPP is a substrate and also comparatively better than GPP with a turnover rate more than double the rate for GPP ($k_{\text{cat}}\text{-NPP} = 0.43 \pm 0.02 \text{ s}^{-1}$, and $k_{\text{cat}}\text{-GPP} = 0.186 \pm 0.002 \text{ s}^{-1}$) and a modest increase in apparent affinity ($K_{\text{M}}\text{-NPP} = 9 \pm 2 \text{ }\mu\text{M}$, and $K_{\text{M}}\text{-GPP} = 13.1 \pm 0.6 \text{ }\mu\text{M}$), resulting in a 3.4-fold increase in catalytic efficiency ($k_{\text{cat}}/K_{\text{M}}\text{-NPP} = 4.7 \times 10^4 \text{ M}^{-1} \text{ s}^{-1}$, and $k_{\text{cat}}/K_{\text{M}}\text{-GPP} = 1.4 \times 10^4 \text{ M}^{-1} \text{ s}^{-1}$) (see Figure 3). The product distribution and enantiomeric purity of the limonene produced in both cases are similar for NPP and GPP (Figures S1 and S2).

FGPP and FNPP as Inhibitors of (+)-LS

The monofluorinated substrate analogues 2-fluorogeranyl and 2-fluoroneryl diphosphate (FGPP and FNPP, respectively) act as competitive inhibitors for the (+)-LS-catalyzed conversion of GPP into limonene (Figure 4). The inhibition constant (K_{I}) for FGPP is $2.4 \pm 0.5 \text{ }\mu\text{M}$, which is similar to the K_{M} for GPP. Despite the clear preference of the enzyme for NPP over GPP as a substrate, FNPP is a weaker inhibitor of the cyclization reaction than FGPP is, with a K_{I} of $39.5 \pm 5.2 \text{ }\mu\text{M}$.

While FGPP and FNPP are effective competitive inhibitors for the reaction of (+)-LS with GPP, we note (as have others) that these fluorinated analogues are not always entirely inert.^{11,21–23} Both FGPP and FNPP are turned over by the enzyme into product, albeit at rates dramatically slower than those observed for GPP (k_{cat} values of 0.00054 ± 0.00005 and $0.00024 \pm 0.00002 \text{ s}^{-1}$ for FGPP and FNPP, respectively). In an overnight reaction, a new peak was detected by GC–MS that was shifted in retention time relative to that of (+)-limonene with a mass fragmentation pattern consistent with a monofluorinated monoterpene (m/z 154 parent ion) (Figure 5).

Structure of (+)-LS with 2-Fluorogeranyl Diphosphate (FGPP)

Crystals of apo-(+)-LS were soaked in solutions of crystallization buffer containing FGPP and MnCl_2 for 1 h before being frozen in liquid N_2 . The structure of FGPP-bound (+)-LS was determined to 2.4 Å resolution using apo-(+)-LS as a search model for molecular replacement. After initial refinement, a difference Fourier density of more than $9\sigma F_{\text{o}} - F_{\text{c}}$ was observed in the active site attached to conserved residues of the metal ion binding sites (Figure 6A; 3σ cutoff shown in the figure). This density was further resolved as three metal ions and a diphosphate based on $F_{\text{o}} - F_{\text{c}}$ peak heights (ranging between 13σ and 17.5σ) as well as interatomic distances, and all three metal ion positions were supported by anomalous difference Fourier peaks [ranging between 11σ and 13σ (data not shown)]. A tail-like density extends from the diphosphate deep into the active site toward the side chain of W315, consistent in length with the prenyl tail of the analogue.

Three Mn^{2+} ions and one molecule of FGPP were modeled into their respective densities. The three metal ions are each bound to six ligands with octahedral coordination geometry, as shown in Figure S3. Mn^{2+}_A coordinates with the two O $\delta 2$ atoms of D343 and D347, O2 α and O2 β of the diphosphate, and two water molecules. Mn^{2+}_B coordinates with O $\delta 1$ of D343, O $\delta 2$ of D347, O2 β of the diphosphate, and three water molecules. Mn^{2+}_C coordinates with O $\delta 2$ of D488, O1 α and O1 β of the diphosphate, and three water molecules. The diphosphate moiety is held firmly between the metal ions, and its position is stabilized by hydrogen bonds from residues R485 and K504 and several water-mediated interactions (Figure 6B).

The prenyl chain of FGPP extends as a left-handed screw deep into the active site. While the conformation about the C2=C3 bond (i.e., rotation about the C1–C2 and C3–C4 bonds) is not unambiguously determined by the electron density, we have modeled the prenyl chain such that *syn* migration of the pyrophosphate to C3 is to the *re* face and would result in the (*R*)-enantiomer of FLPP, as expected for a (+)-LS. Analysis of ligand conformation was performed using Mogul version 1.7.1.²⁴ The geometry of FGPP fragments was found to be mostly within the range of reported structures in the Cambridge Structural Database.²⁵ Outliers reported in the analysis include the angle between the diphosphate moiety atoms Pa, O3 α , and P β and the torsion angle involving these atoms, probably caused by constraints applied to maintain metal ion coordination during refinement. Although strong electron density was not observed for C3, C4, and C10 of the prenyl chain, the analogue seems to adopt a single conformation in the hydrophobic pocket and is held firmly by van der Waals interactions from the side chains of W315, I336, I339, T446, and F484.

Structure of (+)-LS with 2-Fluoroneryl Diphosphate (FNPP)

Crystals of apo-(+)-LS were soaked in solutions of crystallization buffer containing FNPP and MnCl_2 for 1 h before being frozen in liquid N_2 . The structure of FNPP-bound (+)-LS was determined to 2.2 Å resolution using apo-(+)-LS as a search model for molecular replacement. After initial refinement, difference Fourier density ranging between 14 σ and 18.5 σ was observed in the active site (Figure 7A; 3 σ cutoff shown in the figure). Three Mn^{2+} ions and the FNPP ligand were modeled into their respective $F_o - F_c$ peaks.

All three metal ions and the diphosphate moiety are bound to the active-site elements in a configuration similar to that of the FGPP-bound complex (Figure 7B). Analysis of FNPP in this structure shows that the geometry for the FNPP fragments was found to be mostly within the range of reported structures in the Cambridge Structural Database.²⁵ An angle outlier was reported for the diphosphate moiety atoms similar to the case for FGPP as described above. Also, a torsion angle outlier was reported involving the C1–C3 atoms likely due to strain exerted by a lack of density for later atoms of the hydrocarbon chain. Refinement indicated that the prenyl chain of the FNPP analogue could adopt multiple conformations. C7–C9 are held firmly in place through van der Waals interaction with side chains of W315, I336, and F484, while no clear electron density is observed for C3–C5 and C10, suggesting that this stretch of the carbon chain is quite flexible. Two conformations of the analogue were modeled with equal occupancy in the final structure, where the two

conformations diverge most in a hydrophobic patch of the binding pocket formed by the side chains of I339, T340, I445, and T446.

Comparison with the Apoprotein Structure

The overall fold of FGPP- and FNPP-bound (+)-LS differs little from that of the apoprotein (rmsd = 0.3 Å) (Figure S4). The side chain conformation of D347 is different in the ligand-bound forms to coordinate two of the metal ions, but the active site is otherwise little changed. Interestingly, part of the H- α 1 helix and the following loop were found to be completely disordered in the FGPP-(+)-LS structure and partially disordered in the FNPP-(+)-LS structure, suggesting that the ligand is inducing some conformational fluctuation in the protein. We also observe several patches of unaccounted-for electron density in the active site of the ligand-bound (+)-LS structures, consistent with a minor population of the protein trying to adopt the closed conformation but being prevented from doing so by packing forces within the crystal. This conclusion is supported by a comparison with the closed (–)-LS structure from *M. spicata* (*vide infra*). The unaccounted-for electron densities are as follows: near D344 that is occupied by R311 in (–)-LS, near V502 that is occupied by D506 in (–)-LS, near Mn²⁺_C and D488 that is occupied by the loop between the J and K helices in (–)-LS, and, finally, between Mn²⁺_C and T492 that is occupied by T497 alone in the closed conformation of (–)-LS.

DISCUSSION

NPP as a Substrate for (+)-LS

Early experiments surrounding the biosynthetic origins of plant volatile compounds led to many competing hypotheses regarding the universality of GPP as the precursor of all monoterpenes. Stemming from the predicted mechanistic scheme summarized in Figure 8, NPP would appear to be the preferred substrate for monoterpene synthases because of its *cis* arrangement of the carbon backbone around the C2=C3 bond that alleviates the need for bond rotation to bring C1 and C6 into proximity for later ring closure as is “topologically” required for GPP. In the early history of the field, it was determined, however, that GPP is more often the preferred substrate for monoterpene synthases.^{26,27} In our hands, NPP has proven to be a better substrate than GPP for (+)-LS, leading to a >3-fold increase in catalytic efficiency.

NPP was recently shown to be the preferred substrate for a tomato β -phellandrene synthase, and in that case, there was also an extreme difference in enzyme affinity for NPP versus GPP (>300-fold increase in K_M) and a 10¹⁰ loss of catalytic activity with GPP.¹⁹ The specificity of the tomato β -phellandrene synthase for NPP over GPP and the vastly different product profiles generated in both cases led the authors to a mechanistic interpretation in which GPP proceeds through the canonical geranyl cation following ionization and isomerization to LPP but then must proceed through the neryl cation for ring closure to occur. The possibility that the reaction with neryl diphosphate necessarily prevents the generation of the geranyl cation is interesting taken along with our results suggesting that NPP is a better substrate than GPP for (+)-LS.

Fluorinated Substrate Analogues

Fluorine-substituted substrate analogues were first used to explore prenyl transfer reactions by Poulter and co-workers with the enzyme farnesyl pyrophosphate synthetase.^{28–31} Hydrogens at both the C2- and C3-methyl positions of prenyl diphosphates were substituted, ensuring that the electron-withdrawing fluorine atoms were in the proximity of the developing positive charge during ionization of the allylic diphosphates but not in positions where they could stabilize the carbocations through resonance effects. In model studies, S_N1 solvolysis of 2-fluorogeranyl methanesulfonate was shown to proceed 227 times slower than the reaction with nonsubstituted geranyl methanesulfonate, whereas S_N2 displacement of chloride from 2-fluorogeranyl chloride by cyanide was actually enhanced ~ 1.6 -fold in comparison to that of the nonsubstituted geranyl chloride.²⁹ The reaction of farnesyl pyrophosphate synthetase with IPP and GPP proceeded 1200-fold more slowly with FGPP, indicating strongly that the enzyme-catalyzed reaction proceeds stepwise with initial ionization of the allylic diphosphate, as is the case with S_N1 solvolysis of the allylic methanesulfonate model reaction. Given that the van der Waals radius of hydrogen (1.20 Å) is significantly different from that of fluorine (1.47 Å), FGPP is clearly not isosteric with the native GPP substrate. Nonetheless, binding of the analogue to the protein was not greatly affected, as judged by the fact that the K_I for the analogue was similar to the K_M for the nonfluorinated substrates.²⁹

Exploitation of the fluorinated analogues was extended to reactions of monoterpene synthases by Croteau and coworkers, particularly with (–)-LS and (+)-bornyl diphosphate synthase,²¹ culminating in structures for (–)-LS cocrystallized with FGPP and FLPP.¹¹ Interestingly, both structures contained FLPP in the active site but in different conformations. Significantly, the conformation from cocrystallization with FLPP corresponded to a right-handed screw expected of the (–)-LS reaction.

The results reported here for (+)-LS are very much in accord with those reported for farnesyl transferase, (–)-LS, and (+)-bornyl diphosphate synthase noted above. The K_I values for FGPP ($2.4 \pm 0.5 \mu\text{M}$) and FNPP ($39.5 \pm 5.2 \mu\text{M}$) are similar to the K_M values for GPP and NPP (13.1 and $9.1 \mu\text{M}$, respectively), indicating that substitution of a fluorine atom for hydrogen at C2 does not significantly perturb binding of the ligands to (+)-LS. In addition, the large decreases in k_{cat} observed for FGPP (347-fold) and FNPP (1773-fold) are completely consistent with a stepwise mechanism involving ionization of the allylic diphosphate substrate to generate a resonance-stabilized carbenium ion in the rate-limiting step for the enzyme-catalyzed reaction.

FGPP Binding Configuration and Mechanistic Interpretation of Structure

As described in this work, soaking of apoprotein crystals for 1 h with the substrate analogue FGPP or FNPP trapped (+)-LS in a conformation that is intermediate between the open and fully closed forms. We interpret the structures to represent initial binding of the substrate before the enzyme has had a chance to close the binding pocket. In this sense, they are early Michaelis complexes. Taken along with the (–)-LS structural data, this suggests that longer soaking times or cocrystallization will be required to obtain a structure of fully closed (+)-LS.

Comparison of the two ligand-bound structures shows similar interactions with amino acid residues in the active site of the enzyme (Figure 9). The three metal ions and diphosphate occupy essentially identical positions in both ligand-bound structures. In the case of FNPP-soaked (+)-LS, the majority of the carbon chain for the analogue can be traced with sufficient support from electron density, but the region spanning C3–C5 and C10 is absent, implying that this is the most dynamic or least stabilized portion of the analogue. The electron density in the active site is most simply modeled as two alternative configurations of FNPP. In the FGPP-bound structure, the ligand appears to be bound in a single conformation with minor disorder around C4 and C5. Importantly, in neither case, FGPP or FNPP, could the electron density be modeled well with FLPP.

Comparison with Structures of (–)-LS and Bornyl Diphosphate Synthase

The homodimeric crystal structure of FLPP-bound (–)-LS (PDB entry 2ONH) was determined at 2.7 Å resolution by Hyatt et al. after cocrystallization of the ligand and enzyme.¹¹ FLPP is anchored through interaction of the diphosphate with three Mn²⁺ ions and three Asp residues in the active site of the protein in much the same manner as reported for FGPP with (+)-LS here. The amino acid residues that comprise the full ligand binding site (diphosphate and prenyl chain) are identical in the two enzymes or, if different, are at conserved locations in the binding pocket.

A notable difference between the two structures (Figure 10A) is that (–)-LS is in a closed conformation whereas (+)-LS is largely open (presumably as a consequence of crystal contacts preventing conformational transitions after soaking in the ligand). The closed conformation is evident from the N-terminal loop, the H- α 1 helix, and the J–K loop, all covering the active site and giving rise to three differences in the position of conserved residues: T492 is rotated away from the diphosphate in FGPP-(+)-LS due the position of the H- α 1 helix in the open conformation, and its coordination position is replaced by a water molecule; R306, part of the B–C loop, is pointing toward the expected location of the N-terminus in FGPP-(+)-LS; and Y565 is facing away from active site in FGPP-(+)-LS but is pointing toward the ligand in the FLPP-(–)-LS structure.

Structures for two other monoterpene synthases with analogues bound have been reported.^{22,23,32} Because of the similarities, we discuss here only the structure of the well-studied bornyl diphosphate synthase from *Salvia officinalis*.³² The crystal structure of a complex of bornyl diphosphate synthase (BPPS) with the substrate analogue 3-aza-2,3-dihydrogeranyl diphosphate (DHGPP) was determined to be in a closed conformation, similar to that observed for (–)-LS, with the N-terminal loop, H- α 1 helix, and J–K loop closing to exclude the solvent from the active site. The superposition of DHGPP-BPPS and FGPP-(+)-LS active sites (Figure 10B) shows that the positions of conserved active-site residues interacting with analogues and metal ions are identical in both complex structures. The few notable differences between the two complexes are as follows (residues in parentheses correspond to the residue numbering in the BPPS structure): metal ion-coordinating residues T492 (T500) and E496 (E504) in DHGPP-BPPS were replaced by water molecules in the partially closed FGPP-(+)-LS complex; the hydroxyl group of Y565 (Y572) faces the analogue molecule in the DHGPP-BPPS structure, while the whole side

chain is rotated and facing away from active site in FGPP-(+)-LS; two arginine residues in the active site, R306 and R308 (R314 and R316), were well ordered in the BPPS complex but are partially disordered in the FGPP-(+)-LS complex; W315 (W323) appears to be in a different rotamer conformation in both structures, but the plane of the indole side chain facing the ligands is involved in anchoring of the hydrophobic tail in both cases.

Conformation of FGPP in (+)-LS and the Mechanism of Stereoselectivity

We expected initial binding of the GPP substrate to (+)-LS would select for the left-handed screw conformer of the ligand, in complete analogy with the expectation that (–)-LS would select for the right-handed conformer.^{6,7} In agreement with expectations, the prenyl chain in the FGPP structure is in a left-handed screw conformation as predicted for the reaction of (+)-LS with GPP, complementing the results of Hyatt et al.,¹¹ with (–)-LS showing the prenyl chain of bound FLPP to be in a right-handed screw conformation (Figure 10A). While we do not at this point have a definitive explanation for why the two enzymes favor different conformations of the substrates, it is likely that the explanation involves residues M458 of (–)-LS and I336 of (+)-LS. S δ of M458 in (–)-LS [I450 in (+)-LS] would present a steric clash with the terminal methyl groups of FLPP if in a left-hand screw conformation, while C γ 2 of I336 in (+)-LS [N345 in (–)-LS] would present a similar steric clash with the terminal methyl groups of FGPP if in a right-handed screw conformation. Residues I336 and I450 in (+)-LS are the current focus of efforts in our laboratory to understand stereoselectivity in terpene synthases.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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ABBREVIATIONS

GPP	geranyl diphosphate
FPP	farnesyl diphosphate
IPP	isopentenyl diphosphate
LPP	linalyl diphosphate
NPP	neryl diphosphate

FGPP	2-fluorogeranyl diphosphate
FLPP	2-fluorolinalyl diphosphate
FNPP	2-fluoroneryl diphosphate
DHGPP	3-aza-2,3-dihydrogeranyl diphosphate
GC-MS	gas chromatography-mass spectrometry
BPPS	bornyl diphosphate synthase
(+)-LS	(+)-limonene synthase
(-)-LS	(-)-limonene synthase
rmsd	root-mean-square deviation
PDB	Protein Data Bank

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Figure 1.

Stereochemical course expected for the (+)-LS reaction (see also Figure 8). This figure was modified from Scheme 4 of ref².

Figure 2. Chemical structures of the monofluorinated substrate analogues used in this study: (A) 2-fluorogeranyl diphosphate (FGPP) and (B) 2-fluoroneryl diphosphate (FNPP).

Figure 3.

Michaelis–Menten plot for reaction of NPP with (+)-LS. The figure shows a plot of reaction velocity (nanomolar limonene produced per second; ordinate) vs NPP concentration (micromolar; abscissa). Each reaction mixture contained 20 nM (+)-LS, the indicated concentration of NPP substrate (1–200 μM), and 400 μM MnCl_2 . Reactions were performed as described in Experimental Procedures. The reaction for each concentration of NPP was performed in duplicate, where error bars represent the standard deviation. The data were fit to a rectangular hyperbola by nonlinear regression analysis with a K_M of $9 \pm 2 \mu\text{M}$ and a k_{cat} of $0.43 \pm 0.02 \text{ s}^{-1}$.

Figure 4.
Double-reciprocal plots of Michaelis–Menten kinetic data collected in the absence or presence of (A) FGPP and (B) FNPP.

Figure 5.

(A) Gas chromatogram and (B) accompanying mass spectrum for the product of the reaction of FGPP and (+)-LS with Mn^{2+} . In panel A, the data of the (+)-limonene standard are colored black and those of the product red.

Figure 6.

Active-site architecture and electron density for FGPP and metal ions shown in wall-eyed stereoviews. (A) Omit map ($F_o - F_c = 3\sigma$) for FGPP-bound (+)-LS showing electron density for FGPP and three Mn^{2+} ions in the active site of the protein. (B) This model shows the conformation of the bound FGPP and hexacoordination of each of the three Mn^{2+} ions with the diphosphate moiety of the substrate analogue, the conserved aspartates, and water molecules. The diphosphate is stabilized further by hydrogen bonds from basic residues, R485 and K504. For the sake of clarity, only those water molecules coordinated by the metal ions are shown.

Figure 7.

Active-site architecture and electron density for FNPP and metal ions shown in wall-eyed stereoviews. (A) Omit map ($F_o - F_c = 3\sigma$) for FNPP-bound (+)-LS showing electron density for FNPP and three Mn^{2+} ions in the active site of the protein. (B) This model shows two conformations of the bound FNPP and hexacoordination of each of the three Mn^{2+} ions with the diphosphate moiety of the substrate analogue, the conserved aspartates, and water molecules. The diphosphate is stabilized further by hydrogen bonds from basic residues R485 and K504. For the sake of clarity, only those water molecules coordinated by the metal ions are shown.

Figure 8.

Model of limonene cyclization from both GPP and NPP as initial substrates. The scheme was expanded to include passage through the neryl cation, which might help explain why NPP is a better substrate than GPP.

Figure 9. Superposition of the active sites of FGPP-bound (+)-LS (green) and FNPP-bound (+)-LS (gray) showing the conformations of ligand molecules. FGPP and FNPP are color-coded like their respective protein molecules. Mn^{2+} ions of both ligand bound structures are colored purple.

Figure 10.

Superposition of the active sites of FGPP-bound (+)-LS (green) with (A) FLPP-(−)-LS (salmon) (PDB entry 2ONH) and (B) DHGPP-bound BPPS (purple) (PDB entry 1N20) showing conformations of ligands. Ligands and metal ions are color-coded to be the same as the respective protein chains. The G2 helix, J–K loop, and N-terminal loop of FLPP-(−)-LS and the J–K loop and N-terminal loop of BPPS are not shown for the sake of clarity.

Table 1

Crystallographic Data Collection and Refinement Statistics

	LS-FGPP	LS-FNPP
PDB entry	5UV1	5UV2
	Data Collection	
space group		$P4_12_12$
resolution range (Å)	20–2.4	20–2.2
highest-resolution shell (Å)	2.53–2.4	2.32–2.2
unit cell parameters (Å)	$a = b = 85.5, c = 215.4$	$a = b = 85.7, c = 214.9$
total no. of reflections	851665	1149936
no. of unique reflections	31630	41589
completeness (%) ^a	98.7 (98.3)	99.9 (100)
R_{merge} (%) ^a	15.1 (207)	11.7 (212)
$\langle I \rangle / \sigma(I)$ ^a	14.5 (2.2)	22 (2.1)
CC(1/2) (%) ^a	99.9 (85.9)	100 (74.2)
redundancy ^a	26.9 (27.7)	27.7 (28.5)
	Refinement	
resolution range (Å)	20–2.4	20–2.2
no. of reflections used	31273	41492
R_{cryst} (%)	20.3	19.3
R_{free} (%)	23.6	23.0
no. of protein atoms	4212	4261
no. of ligand atoms	20	20
no. of metal atoms	3	3
no. of water molecules	144	201
rmsd for bond lengths (Å)	0.002	0.008
rmsd for bond angles (deg)	0.4	1.0

^aHighest-resolution shell values are given in parentheses.