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Functional and Structural Characterization of a (+)-Limonene Synthase from *Citrus sinensis*

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Abstract

Terpenes make up the largest and most diverse class of natural compounds and have important commercial and medical applications. Limonene is a cyclic monoterpene (C₁₀) present in nature as two enantiomers, (+) and (–), which are produced by different enzymes. The mechanism of production of the (–)-enantiomer has been studied in great detail, but to understand how enantiomeric selectivity is achieved in this class of enzymes, it is important to develop a thorough biochemical description of enzymes that generate (+)-limonene, as well. Here we report the first cloning and biochemical characterization of a (+)-limonene synthase from navel orange (*Citrus sinensis*). The enzyme obeys classical Michaelis–Menten kinetics and produces exclusively the (+)-enantiomer. We have determined the crystal structure of the apoprotein in an “open” conformation at 2.3 Å resolution. Comparison with the structure of (–)-limonene synthase (*Mentha spicata*), which is representative of a fully closed conformation (Protein Data Bank entry 2ONG), reveals that the short H- α 1 helix moves nearly 5 Å inward upon substrate binding, and a conserved Tyr flips to point its hydroxyl group into the active site.

Graphical abstract

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Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.biochem.7b00143. Protein sequence alignment (Figure S1), Purification of (+)-LS as followed by SDS–PAGE and SEC (Figure S2), Divalent metal cation dependence of activity (Figure S3), Apo-(+)-LS and (–)-LS comparative overlay (Figure S4), Michaelis-Menten plot for reaction of GPP with (+)-LSY565F (Figure S5), and Gas chromatogram for reaction of the Y565F mutant with GPP (Figure S6) (PDF)

Accession Codes

The nucleotide sequence for full-length (+)-LS from *C. sinensis* (navel orange) has been deposited as GenBank accession code KU746814. The atomic coordinates and structure factors for apo-(+)-LS have been deposited as PDB entry 5UV0.

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Notes

The authors declare no competing financial interest.

Terpenes make up the largest and most structurally diverse class of natural products.¹ They are secondary metabolites involved in a host of functions in many different biological systems. Plants in particular have developed a diverse array of terpenes that serve as signaling hormones, as chemical defense agents against microbial infection and predation, and as attractants for pollinators.¹ Terpenes also play important industrial roles as solvents, fragrances, flavorings, and materials, and many have pharmaceutical applications. Despite their structural and functional diversity, most terpenes are derived from only three simple acyclic precursors: C₁₀ monoterpenes from geranyl diphosphate (GPP), C₁₅ sesquiterpenes from farnesyl diphosphate (FPP), and C₂₀ diterpenes from geranylgeranyl diphosphate (GGPP).

The committed step in terpene biosynthesis generally involves conversion of the acyclic isoprenoid diphosphate precursor to cyclic hydrocarbon products through the action of enzymes known as terpene synthases (or cyclases).^{2–7} The divalent metal ion (typically Mn²⁺ or Mg²⁺) dependent reaction in class 1 cyclases⁸ is initiated by ionization of the diphosphate precursor to generate a high-energy, allylic carbenium ion intermediate.⁹ The synthase then controls the reactivity of this intermediate to generate a vast array of different products that can result from carbon skeleton rearrangements as well as methyl and hydride shifts.⁹

One of the simplest cyclization reactions is catalyzed by limonene synthase (Figure 1).⁶ Limonene is a cyclic monoterpene that is present in nature as two enantiomers, (+) and (–), which are produced by different enzymes. The proposed cyclization reaction for limonene synthase (as for all monoterpene synthases) begins with stereoselective binding of GPP to the enzyme as a right-handed or left-handed conformer, depending on the stereochemistry of the resulting product.¹⁰ The diphosphate moiety then dissociates, forming a resonance-stabilized carbenium ion intermediate. The diphosphate is believed to reattach at the tertiary C3 to form either (3*R*)- or (3*S*)-linalyl diphosphate (LPP), allowing rotation about the C2–

C3 bond to place C1 in a position permitting cyclization with C6. All monoterpenes are thought to proceed through the common α -terpynyl cation, and the resulting skeletal diversity of the monoterpenes is dictated by termination of the reaction, which can involve deprotonation of a methyl group (as is the case for limonene) or nucleophilic attack by water (as is the case for terpineol) followed by release of the cyclic product.⁶

The crystal structure of a (–)-limonene synthase [(–)-LS] has been determined previously after cocrystallization with the substrate analogues 2-fluoro-GPP (FGPP) and 2-fluoro-LPP (FLPP) [Protein Data Bank (PDB) entries 2ONG and 2ONH, respectively].¹¹ These structures revealed that (–)-LS shares many of the hallmark features characteristic of plant monoterpene synthases, including an all- α -helical domain secondary structure, a two-domain architecture with a catalytic C-terminal domain and an N-terminal domain of unknown function, and conserved divalent metal ion binding residues in the active site. Although (–)-LS was cocrystallized with FGPP, electron density in the active site was better fit by modeling in FLPP in an extended configuration, implying that the structure represents a snapshot of the reaction in progress.

We report here the cloning and biochemical characterization of a (+)-limonene synthase [(+)-LS] from navel orange (*Citrus sinensis*). (+)-Limonene is a highly abundant monoterpene and is the principle terpene constituent of most citrus fruit essential oils.¹² While it is commonly used as an industrial and household solvent, its characteristic citrus smell has also made it particularly useful for the fragrance and flavoring industries, and it has noted potential as a renewable biofuel.¹³ We present the crystal structure of the apoprotein determined at 2.3 Å resolution in an “open” conformation. Comparison of our apo-(+)-LS structure with that of FLPP-bound (–)-LS highlights conformational changes that are likely important for the transition from open apoenzyme to the fully closed substrate-bound form.

EXPERIMENTAL PROCEDURES

Synthesis of Geranyl Diphosphate

GPP was synthesized from geraniol using the large-scale phosphorylation procedure previously described by Keller and Thompson in which geraniol (300 mg) was phosphorylated by reaction with triethylammonium phosphate (TEAP) and trichloroacetonitrile at 37 °C.¹⁴ The completed reaction mixture was stored overnight at –20 °C and then 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 developed in a 6:3:1 isopropanol/ammonium hydroxide/water mixture and visualized with KMnO₄. Fractions containing pure GPP were pooled, concentrated by rotary evaporation at reduced pressure, flash-frozen in liquid nitrogen, and lyophilized to dryness for 18–24 h.

Lyophilized GPP was further purified by anion exchange chromatography using DOWEX-1X2-400 strongly basic anion exchange resin chloride form (Sigma). A 1 cm × 8 cm resin bed was equilibrated with 125 mM ammonium bicarbonate (pH 8), loaded with 10–30 mg of GPP, and washed with 40 mL of 125 mM ammonium bicarbonate at a flow rate of

2 mL/min. The column was then eluted using 500 mM ammonium bicarbonate. Fractions were analyzed on TLC plates and visualized with KMnO_4 to locate the eluted GPP that was then flash-frozen in liquid nitrogen and lyophilized to dryness for 24–48 h. The lyophilized product was stored at -20°C until it was needed. The purity was assessed by proton, carbon, and phosphorus nuclear magnetic resonance (NMR) spectroscopy. NMR spectra were recorded on a Varian 400-MR spectrometer (9.4 T, 400 MHz) in D_2O adjusted to pH ~8.0 with ND_4OD . ^1H and ^{13}C chemical shifts are reported in parts per million downfield from TMSP (trimethylsilyl propionic acid), and ^{31}P chemical shifts are reported in parts per million relative to 85% α -phosphoric acid. 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): ^1H NMR (400 MHz, $\text{D}_2\text{O}/\text{ND}_4\text{OD}$) δ_{H} 1.64 (3 H, s, CH_3), 1.70 (3 H, s, CH_3), 1.73 (3 H, s, CH_3), 2.08–2.20 (4 H, m, H at C4 and C5), 4.48 (2 H, app t, $J = 6.6$ Hz, $J_{\text{H,P}} = 6.6$ 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); $^{13}\text{C}\{^1\text{H}\}$ NMR (100 MHz, $\text{D}_2\text{O}/\text{ND}_4\text{OD}$) δ_{C} 18.45, 19.81, 27.67, 28.45, 41.64, 65.32 (1 C, d, $J_{\text{C,P}} = 5.34$ Hz), 122.90 (1 C, d, $J_{\text{C,P}} = 8.39$ Hz), 127.03, 136.57, 145.43; $^{31}\text{P}\{^1\text{H}\}$ NMR (162 MHz, $\text{D}_2\text{O}/\text{ND}_4\text{OD}$) δ_{P} -5.74 (1 P, d, $J_{\text{P,P}} = 22.1$ Hz, P1), -9.55 (1 P, d, $J_{\text{P,P}} = 22.1$ Hz, P2).

Cloning of the *C. sinensis* (+)-Limonene Synthase Gene

Total RNA was extracted from the flavedo of a navel orange (*C. sinensis*), purchased at a local supermarket, using the RNeasy Plant Mini kit (Qiagen) under conditions recommended for recalcitrant material. First-strand cDNA synthesis with reverse transcriptase was performed using the Super Script III CellsDirect cDNA Synthesis System (Thermo Fisher Scientific). Double-stranded cDNA for the limonene synthase gene was amplified using primers based on the sequence of the published *Citrus limon* (+)-LS gene (GenBank accession number AF514287), which we expected to be similar to the gene found in navel orange.¹⁵ The following primers were used: 5'-CCG tat aag ccg gtc gac g atg tct tct tgc ATT AAT CCC TCA ACC TTG-3' and 5'-AAA TGA GCG GCC GC TCA GCC TTT GGT GCC AGG AGA TGC TGT-3'. A *SaI* site was introduced at the 5'-end and a *NotI* site at the 3'-end to facilitate recovery of DNA from the vector.

Amplified cDNA was cloned into a pCRII-TOPO vector using the *TOPO TA Cloning* kit (Thermo Fisher Scientific). The vector was transformed into chemically competent TOP10F' cells that were then grown overnight at 37°C on LB/agar plates containing 100 $\mu\text{g}/\text{mL}$ ampicillin, 40 $\mu\text{g}/\text{mL}$ X-gal, and 100 mM isopropyl β -D-1-thiogalactopyranoside (IPTG). Following blue/white colony screening, colony PCR (using the same primers as used for RT-PCR) was performed to confirm the presence of the gene. The nucleotide sequence has been added to GenBank (accession code KU746814). The cDNA was then truncated at the 5'-end (nucleotides encoding amino acids 1–52) to remove the plastid targeting sequence, modified by addition of an N-terminal His₆ tag for purification, and cloned into the *NcoI* and *NotI* restriction enzyme-cut sites of a pET-28a (+) expression vector.

Site-directed mutagenesis was performed to substitute the Tyr at position 565 with Phe (Y565F) using the QuikChange II Site-Directed Mutagenesis Kit (Agilent Technologies). The following mutagenic primers were designed: Y565F, 5'-GTC CCA TTT TAT GTT TCT

ACA TGG AG-3' and 5'-CTC CAT GTA GAA ACA TAA AAT GGG AC-3'. The nucleotide substitution was verified by sequencing the full construct (Genewiz).

Protein Expression and Purification

The N-terminally His₆-tagged and truncated (+)-LS construct was subcloned into the pET-28a (+) vector and transformed into BL21-CodonPlus(DE3)-RIL cells (Agilent Technologies). The transformed cells were selected on LB/agar plates containing 50 µg/mL kanamycin and 20 µg/mL chloramphenicol following incubation at 37 °C overnight. Single colonies were selected and grown in small overnight cultures (10 mL of LB, again with 50 µg/mL kanamycin and 20 µg/mL chloramphenicol) at 37 °C with 220 rpm shaking overnight. One milliliter of the overnight culture was used to inoculate 1 L of LB (in the absence of antibiotics) and grown at 37 °C until the OD₆₀₀ was between 0.5 and 0.8, at which time protein expression was induced by addition of IPTG to a final concentration of 1 mM. The temperature was decreased to 20 °C, and after being induced for 20 h, cells were pelleted by centrifugation. Pelleted cells were resuspended in 20 mL of lysis buffer [50 mM Tris (pH 7.5), 100 mM NaCl, and 20 mM imidazole] and stored at -80 °C until they were needed.

Frozen cell suspensions were thawed on ice and brought to 50 mL with lysis buffer. DNase I and lysozyme were each added to a final concentration of 10 µg/mL along with five EDTA-free Pierce protease inhibitor cocktail tablets (Thermo Fisher Scientific). The cell suspension was sonicated on ice using a preset cycle of 20 s on and 30 s off for a total of 3 min on-time at roughly 50–100 mW power. Cell debris was pelleted by centrifugation at 9490 rcf for 45 min at 4 °C, and the supernatant fraction was passed through a 0.22 µm filter and then loaded at a rate of 1 mL/min onto a prepacked 5 mL HiTrapFF Ni-Sepharose column (GE Healthcare Life Sciences) that had been previously equilibrated in lysis buffer. The column was washed at a rate of 1.5 mL/min with 40 mL of wash buffer (lysis buffer with the imidazole concentration increased to 40 mM) and then eluted with an 80 mL linear gradient ranging from 40 to 500 mM imidazole also at a rate of 1.5 mL/min. Fractions containing (+)-LS were identified via sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and pooled before being concentrated with an exchange of buffer [50 mM Tris (pH 7.5) and 100 mM NaCl] to remove imidazole (50 kDa molecular weight cutoff Amicon Ultra Centrifuge Filter from EMD Millipore).

Size-Exclusion Chromatography

Size-exclusion chromatography (SEC) was conducted using an Äkta FPLC system (Amersham Biosciences, Uppsala, Sweden) equipped with a Superdex-200 10/300 GL gel filtration column (GE Healthcare Life Sciences); 300 µL of 100 µM (+)-LS was loaded onto the column that was equilibrated in 50 mM Tris (pH 8), 100 mM NaCl, and 10% glycerol at 4 °C. The flow was maintained at a constant rate of 0.5 mL/min. The void volume was measured at 8.3 mL by eluting blue dextran as a high-molecular weight standard (MW ~ 2000 kDa). The total internal column volume was measured at 21.19 mL with vitamin B₁₂ as a low-molecular weight standard (MW = 1.35 kDa). These and other well-resolved molecular weight standards were used to derive a standard curve that allowed accurate estimation of the molecular weight of eluted (+)-LS.

Enzymatic Activity Assay

The enzymatic activity was monitored using the discontinuous single-vial assay described by O'Maille et al.¹⁶ with the exception that hexane was used for the organic layer rather than ethyl acetate. Each screw-cap vial contained a 1 mL reaction mixture composed of 50 mM Tris (pH 8.0), 100 mM NaCl, 10% (v/v) glycerol, the purified protein, GPP, and MnCl₂ (400 μ M) overlaid with 1 mL of hexane. Reactions were initiated by addition of substrate and allowed to proceed for various times, and mixtures were vortexed for 30 s to both terminate the reaction and extract terpene products into the organic layer. The progress of the reaction 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. Reactions were measured under initial rate conditions (linear time course from 2 to 6 min) for a range of substrate concentrations (1–200 μ M) under conditions that were also linear with enzyme concentration. The resulting velocity versus substrate concentration data were fit by nonlinear regression (Igor Pro software package, WaveMetrics) with the Michaelis–Menten equation [$v = (V_{\max}[S])/(K_M + [S])$] to extract the kinetic parameters K_M and k_{cat} .

Gas Chromatography and Mass Spectrometry

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 inject 5 μ L samples onto an HP-5ms (5%-phenyl)-methylpolysiloxane capillary GC column (Agilent Technologies, 30 m $\times$ 250 μ m $\times$ 0.25 μ m) at an inlet temperature of 220 °C and 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 at 50 °C/min to a final temperature of 240 °C, which was then held for 1 min. Retention times coupled with mass spectra were verified using commercially available terpene standards.

Circular Dichroism

Limonene enantiomeric purity was determined by circular dichroism (CD). CD experiments were conducted using a J-810 spectropolarimeter (Jasco). Limonene was dissolved in hexanes, and measurements were taken using a 1 mm path-length quartz cuvette. A total of 15 accumulations were recorded for each sample. Spectra were recorded from 300 to 185 nm at a bandwidth of 1 nm and a scan speed of 100 nm/min. All spectra were recorded at 25 °C. The enantiomeric purity was determined by comparing the molar ellipticity ($[\theta]$, deg $\cdot$ cm² $\cdot$ dmol⁻¹) of the enzymatic sample with that of a commercially sourced (+)-limonene standard (Sigma).

Crystallization

Crystallization trials were set by mixing 15 mg/mL protein in buffer [50 mM Tris (pH 7.5) and 100 mM NaCl] with mother liquor at a 1:1 (v/v) ratio. Initial trials were performed using the sitting drop vapor diffusion method with Jena Bioscience sparse matrix crystallization screens (Jena Bioscience, Jena, Germany). Drops were set with the aid of a Phoenix

crystallization robot (Art Robbins Instruments). The hanging drop method was used for further optimizations. Crystals approximately 0.3 mm × 0.4 mm × 0.2 mm in size grew in the presence of 12–16% PEG-8000, 100 mM Tris (pH 7.5–9.0), and 200–350 mM sodium tartrate after 10 days at 20 °C.

Data Collection, Processing, and Refinement

Crystals were flash-frozen in liquid nitrogen after being soaked briefly in mother liquor containing 20% glycerol. Data sets were collected at beamline 8.2.1 at the Advanced Light Source (Lawrence Berkeley National Laboratory, Berkeley, CA) using ADSC Q315R CCD detectors (Area Detector Systems Corp.) at a temperature of 100 K. The best crystals diffracted to 2.3 Å resolution. The data were indexed and integrated using iMosflm version 7.2¹⁷ and scaled using SCALA version 3.3¹⁸ from the CCP4 software suite version 6.4.^{19,20} Diffraction data were processed in the $P4_12_12$ space group. The unit cell dimensions were as follows: $a = b = 85.8$ Å, $c = 216.4$ Å, and $\alpha = \beta = \gamma = 90^\circ$. Complete data collection statistics are listed in Table 1.

The structure of (+)-LS was determined by molecular replacement with PHASER²¹ using the structure of (–)-LS from *M. spicata* as a search model (PDB entry 2ONG). The molecular replacement solution found one protein monomer in the asymmetric unit. The structure was initially refined to starting R and R_{free} values of 0.238 and 0.282, respectively. Refinement was performed using the function phenix.refine²² in the PHENIX software suite version 1.10²³ with the initial two positional refinements preceded by rigid body refinement. All model building was done using COOT version 0.8.²⁴

The altered position of the H- α 1 helix in the apoprotein structure was rebuilt from the original model, and 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 model. Spherical solvent peaks above $3\sigma F_o - F_c$ and $1\sigma 2F_o - F_c$ were identified, and water molecules were modeled in and included in the final rounds of refinement. The final structure was refined to R and R_{free} values of 0.190 and 0.229, respectively. The refinement statistics are listed in Table 1. Coordinates and structure factors for the apoprotein (PDB entry 5UV0) data set have been deposited in the Protein Data Bank. All crystal structure figures in this paper were prepared using PyMol version 1.3 (Schrödinger LLC, Portland, OR).

RESULTS

(+)-LS, the (+)-Limonene Synthase from Navel Orange

cDNA for (+)-LS was obtained from the flavedo of a navel orange (see Experimental Procedures). The translated protein sequence consists of 607 amino acids (including Nterminal methionine) with an estimated molecular weight of 70.4 kDa, which places it in the range of other plant monoterpene synthases that typically have two similarly sized domains: an N-terminal domain of unknown function and a C-terminal domain that contains the active site.⁷ Sequence elements shared by other monoterpene synthases are conserved in (+)-LS, including the plastidial targeting sequence, the Nterminal Arg pair of the mature protein, and acidic divalent metal ion binding sites. Plant synthases often bear a signature N-

terminal plastidial targeting sequence of approximately 50–60 residues that is noted for being rich in small polar residues (Ser and Thr) and devoid of charged acidic residues (Asp and Glu) but is otherwise without identifiable sequence conservation.²⁵ The targeting sequence is removed by proteolysis once the synthase has been transported into chloroplasts, and the mature, functional form of the protein is thought to begin with a pair of conserved Arg residues (R53 and R54) at the Nterminus, which are involved in closing off the active site to solvent once substrate has bound. The divalent metal cation binding sites, [DDXXD] and [(N/D)DXX(S/T)XXXE],⁴ found in the D and H helices, are also present in the orange (+)-LS (D343, D344, D347 and D488, D489, T492, and E496).

A BLAST search of (+)-LS identified the (+)-limonene synthase CitMTSE2 from satsuma mandarin orange (*Citrus unshiu*)²⁶ as the closest homologue with 99.7% sequence identity. The two sequences differ at only two positions, both in the noncatalytic N-terminal domain: a conservative substitution of Val for Ala and an insertion of a Lys residue in the mandarin orange sequence. The next closest homologue is the (+)-limonene synthase C1(+)-LIMS2 from lemon (*C. limon*)¹⁵ with 95.6% amino acid identity, where again most of the sequence differences are in the N-terminal domain. In contrast to the high degree of sequence identity among the citrus fruit (+)-limonene synthases, the sequence of (+)-LS is only 44.7% identical to that of the (–)-LS from spearmint (*M. spicata*),²⁷ although the residues lining the active site are mostly conserved (Figure S1; see also Figure 7).

Purification

Recombinant expression of plant terpene synthases has been shown to be improved by truncation of the full-length proteins to remove the N-terminal plastidial targeting sequence up to the tandem pair of Arg residues. Further truncation results in a dramatic loss of activity.^{28,29} Truncation improves purification because the full-length protein often is strongly associated with *E. coli* chaperone proteins, and the majority of the expressed protein is sequestered in inclusion bodies. The (+)-LS gene was modified to replace codons of the plastidial target sequence (amino acid residues 1–52, inclusive) with a His₆ tag (used for purification) that was then followed by the tandem Arg pair, R53 and R54, and remaining coding sequence for the mature protein. (+)-LS was transformed into BL21-CodonPlus(DE3)-RIL cells (Agilent Technologies) that contain extra copies of the tRNAs that recognize the infrequently used codons for Arg, Leu, and Ile (often found in plant terpene synthase genes). The protein was purified using immobilized nickel affinity chromatography resulting in roughly 10–20 mg of soluble protein/L of cells and >90% purity as judged by SDS-PAGE (Figure S2A). FPLC SEC on Superdex-200 showed predominantly a single peak corresponding to a molecular weight of ~70 kDa that is consistent with the molecular weight expected for a monomer (562 amino acids; MW = 65.7 kDa) (Figure S2B).

Activity

Initially we tested both GPP (for synthesis, see Experimental Procedures) and farnesyl diphosphate (FPP) (Sigma) as possible substrates for (+)-LS. A discontinuous *in vitro* single-vial assay was used to monitor enzyme activity.¹⁶ In a 1 mL reaction mixture, the divalent cation (Mn²⁺ or Mg²⁺), substrate, and enzyme were combined and overlaid with an

equal volume of hexanes. The reaction mixture was incubated overnight before the reaction was quenched with vigorous mixing for 30 s and extraction of hydrophobic compounds into the organic phase, which was then loaded directly onto the GC-MS instrument. Reaction products were identified on the basis of the measured fragmentation patterns generated by the mass spectrometer in comparison to commercially available terpene standards. Incubation with FPP resulted in no detectable product, even after several days (data not shown). In Figure 2A, the gas chromatogram for the reaction with GPP clearly shows the production of limonene as the major species (99% total product). Smaller amounts of the monoterpenes β -myrcene (0.7%) and α -pinene (0.3%) were also detected. Terpene synthases are known to be capable of producing multiple terpene products;³⁰ however, (+)-LS appears to exhibit a high degree of control over product outcome and generates mainly a single product.

In a control experiment, no hexane extractable terpene products were produced in the absence of divalent metal ion. The enzyme generated the same distribution of terpene products in the presence of either magnesium or manganese; however, the maximal rate of turnover differed (Figure S3). The concentration at which the enzyme reached optimal activity was significantly lower for Mn^{2+} (600 μM) than for Mg^{2+} (20 mM), and Mg^{2+} at saturating levels was capable of producing limonene at only ~60% of the rate for optimal Mn^{2+} . Additionally, enzyme activity increased gradually with increasing magnesium concentration until it reached a point of saturation (above 20 mM), whereas enzyme activity was acutely affected by even small increases in manganese concentration and was strongly inhibited at concentrations of >1 mM. This inhibitory effect has been observed for other terpene synthases, although the mechanism behind it remains undetermined.³¹ In mixed metal experiments, the inhibitory effects of millimolar Mn^{2+} were still observed even at saturating Mg^{2+} concentrations. Some terpene synthases have also been shown to be dependent on monovalent cations, specifically K^+ , for activity.³² However, when tested, no effect on the (+)-LS reaction rate was observed with either potassium or sodium at concentrations from 0 to 300 mM. Above 300 mM, activity sharply declined likely because of nonspecific effects of ionic strength.

Circular dichroism measurements were taken to determine the enantiomeric selectivity of (+)-LS. CD spectra for control samples of pure (+)- and (–)-limonene were compared with those of enzymatically produced limonene. Enantiopure samples of (+)- and (–)-limonene generated characteristic spectra with distinguishable Cotton effects. Spectra for the enzymatic product and (+)-limonene standard were nearly superimposable after correction for differences in concentration. The results of these experiments show not only that (+)-LS produces the (+)-enantiomer but also that the reaction is stereospecific in that it produces negligible if any of the (–)-enantiomer within the limits of detection using this method (Figure 2B). These results are in agreement with those published for the nearly identical (+)-LS from *C. unshiu* in which it was shown using separation on a chiral GC column that the enzyme made exclusively the (+)-enantiomer.²⁶

Enzymatic activity was also measured by following the production of limonene over time while varying the concentration of substrate. The enzyme exhibited classical Michaelis-Menten saturation kinetics where reaction rates plotted against substrate concentration were

fit well by the equation $v = (V_{\max}[S])/(K_M + [S])$. From these data (Figure 3), it was possible to extract a K_M of $13.1 \pm 0.6 \mu\text{M}$ and a k_{cat} of $0.186 \pm 0.002 \text{ s}^{-1}$. Both numbers fall within the typical range observed for other terpene synthases in the literature. No substrate inhibition was observed in the range of GPP concentrations tested, unlike what had been previously reported for the (+)-LS from lemon.¹⁵ The same experiment was conducted substituting magnesium for manganese, and consistent with the metal ion dependency data, the catalytic turnover appeared to be reduced by ~40%; the K_M was also shifted by ~3-fold ($k_{\text{cat}} = 0.118 \pm 0.005 \text{ s}^{-1}$, and $K_M = 35 \pm 6 \mu\text{M}$).

Structure of Apo-(+)-LS

Apo-(+)-LS crystallized from PEG-8000 solutions in space group $P4_12_12$ with one molecule in the asymmetric unit. The structure was determined by molecular replacement using (–)-LS from *M. spicata* as a search model (PDB entry 2ONG) and refined to 2.3 Å resolution. Electron density was weak for residues of the N-terminus up to and including Q59, the T219-E225 loop, the Q574-E577 loop, and residues of the C-terminus following L592, and for this reason, these residues were not included in the final structure. As shown in Figure 4, the protein is composed of two domains typical of plant monoterpene synthases: an N-terminal domain of unknown function and a C-terminal domain responsible for catalytic activity. The catalytic domain displays the classic “terpene synthase fold” composed of 12 helices.^{33,34} Six (C, D, F, G1–G2, H2–H- α 1, and J) of the 12 helices form the walls of the active site cavity that is lined with mostly nonpolar, hydrophobic, and aromatic amino acid residues³⁵ and is flanked on either side by the metal ion binding residues D343, D347, and D488 that are part of conserved motifs [DDXXD] and [(N/D)DXX(S/T)XXXE].⁴ In contrast to the other apo structures of monoterpene synthases, bornyl diphosphate synthase³⁵ and 1,8-cineole synthase,³⁶ (+)-LS displays clear electron density for the H- α 1 helix. The H- α 1 helix is believed to be involved in closing the active site to solvent upon binding of substrate.

We observe unaccounted-for electron density deep in the active site that cannot be modeled with GPP substrate, limonene product, buffer molecules, or other compounds present in the crystallization solutions, and no metal ions are found bound to the protein. This density was not present when crystals were soaked with fluorinated substrates (DOI: 10.1021/acs.biochem.7b00144).

Comparison of Apo-(+)-LS with FLPP-Bound (–)-LS

Superposition of the structures of apo-(+)-LS, described here, and FLPP-bound(–)-LS (PDB entry 2ONG)¹¹ shows no significant differences in overall fold with an rmsd ($C\alpha$ atoms) of 1.4 Å, despite the fact that the sequences of these two proteins are only 44.7% identical (Figure S4). However, there are a few differences in the catalytic C-terminal domain that likely can be ascribed to differences between an open active site conformation [our apo-(+)-LS structure] and a closed, ligand-bound conformation [the FLPP-bound (–)-LS structure]. Most notable is an ordering of the N-terminal strand and loop between the J and K helices, which together form a lid covering the active site in the (–)-LS structure while the short H- α 1 helix shifts ~5 Å inward to cover the active site from below (Figure 5). The H- α 1 helix is stabilized in the open conformation by a series of hydrogen bonds with residues on helix I (S494 O γ ···E517 O ϵ , T492 O···R521 NH2, and L490 O···R521 NH1)

(Figure 6A) that are lost upon the transition to the closed conformation. T492 reorients to coordinate Mn^{2+}_C in the ligand-bound conformation; P503 acts as a hinge that allows movement of the H- α 1 helix, and a hydrogen bond between the carbonyl oxygen of V502 and N ϵ 2 of Q507 is broken, resulting in relocation of V502 and an $\sim 180^\circ$ rotamer flip of the Q507 side chain (Figure 6B). Two final changes of note in the closed conformation are reorientation of D347 to coordinate two of the Mn^{2+} ions (Figure 5) and movement of the phenolic side chain of Y565 in the J helix from a position outside of the active site to a position in the active site with the -OH group pointing toward the ligand (Figures 5).

The J helix is a half-turn longer, and the loop between the J and K helices is outwardly projected in the apo structure (Figures 5). Upon substrate binding, the loop between the J and K helices moves to cover the active site and partially unwinds the J helix by a half-turn up to Y565. This unwinding event appears to trigger the side chain of Y565 to flip inward toward the active site. The hydroxyl group of the Y565 side chain [Y573 in (-)-LS] comes within hydrogen bonding distance (2.8 Å) of O δ 1 in the bidentate carboxylate side chain of the manganese-coordinating D488 [D496 in (-)-LS] and is 3 Å from C2 of the prenyl chain of FLPP in the (-)-LS structure. To investigate the role of Y565 in catalysis, the residue was mutated to Phe (Y565F), preserving the aromaticity and hydrophobicity of the side chain while removing its hydrogen bonding capability. The Y565F mutant was expressed at levels similar to that of the wild-type protein but was found to have a reduced activity with a k_{cat} of 0.004 s^{-1} and a modestly smaller K_M of $4.6\text{ }\mu\text{M}$ for GPP (see Figure S5). These data suggest that the phenolic side chain may indeed move into the active site upon substrate binding and that this transition is important for the full activity of the enzyme. Additionally, while the major product formed by the reaction of the Y565F mutant of (+)-LS with GPP was still limonene, oxygenated monoterpenes (mainly isomers of terpineol) were also produced in smaller proportions as was the isomerized hydrolysis product of GPP, linalool (Figure S6). The wild-type enzyme does not generate any oxygenated products. The transition to the closed active site conformation is likely critical to excluding solvent molecules from the active site that might react with high-energy carbenium ion intermediates along the reaction coordinate.

DISCUSSION

Citrus Fruit History and Sequence Differences

Citrus fruit evolutionary history has been difficult to assess comprehensively.³⁷ Oranges in particular have become heavily cross-bred, which makes it difficult to trace the origins of any one species. Most of the differences between subspecies of oranges are phenotypic (i.e., thicker rind and sweeter juice) and these qualitative traits are likely to be differently interpreted worldwide, resulting in many names for many different cultivars, which further convolutes the tracing of their agricultural history.³⁷ Branch grafting onto host citrus trees also means that some trees may be producing multiple different species of fruit at the same time.

Our gene for (+)-LS came from a navel orange purchased from a local supermarket. Navel oranges are a popular subvariety of sweet oranges (*C. sinensis*) noted for their thick skin, lack of seeds, and signature navel, which is the result of a second immature fruit forming at

the base of the orange. Sequence alignment with other reported (+)-limonene synthases identified the gene product CitMTSE2 from satsuma mandarin orange (*C. unshiu*) as the closest homologue (99.7% identical). These two orange subspecies are vastly different in morphology, yet the limonene synthase gene has not seen robust changes in sequence. As navel oranges were first recognized only 200 years ago, it is possible that the two orange subtypes have not had enough time for their genomes to diverge significantly.³⁸ In a recent genome sequencing effort for *C. sinensis* (cv. Valencia), it has been proposed that the sweet orange as we know it today is the result of a more ancient hybrid backcross between a pummelo and a mandarin, suggesting that the satsuma mandarin and the navel orange might be more closely related than their difference in appearance implies.³⁹

Enantiomeric Selectivity

A surprising result of this structural investigation has been that two terpene synthases somehow evolved ways of selecting for the production of one enantiomer of the same terpene skeleton over another even though they share similar global protein folds. Although (–)- and (+)-LS produce two different enantiomeric compounds, there appear to be very few differences in amino acid residues present in the active site that may confer this selectivity (Figure 7). Enantiomeric selectivity in these enzymes is thought to be conferred by the initial binding configuration of the substrate.^{2,6} Support for this hypothesis is provided by a crystal structure for (+)-LS bound to the fluorinated substrate analogue 2-fluoro GPP, presented in the following article in this issue (DOI: 10.1021/acs.biochem.7b00144).

Open to Closed Structural Differences

Despite sharing only 44.7% sequence identity, the structures of apo-(+)-LS and FLPP-bound (–)-LS show few significant differences in the overall fold of the proteins (C α atom rmsd of 1.4 Å). Differences between the structures near the active site can thus be considered to be representative of the conformational changes associated with substrate binding. This is especially true for the N-terminal strand, J–K loop, and H- α 1 helix. What might the structural differences observed between our apo structure and the analogue-bound structure of (–)-LS from *M. spicata* mean for the mechanism of monoterpene cyclization? As we follow the structures from open to closed, we observe the inward motion of the H- α 1 helix, similar to what is observed in other terpene synthases.^{35,36} It is believed that this short helix acts to close off the active site from solvent molecules, protecting the highly reactive carbocations from premature quenching by water. It appears that the movement of this helix is triggered by the breaking and making of only a few hydrogen bonds driven by the coordination of Mn²⁺_C by T492. The inward movement of the H- α 1 helix is likely to be coupled to the capping of the active site by the N-terminal strand, stabilized at one end by the tandem pair of Arg and at the other end by an invariant Trp residue (W63) that forms hydrophobic packing interactions above the active site.

The Tyr at position 565 on the J helix appears to undergo a transition from a position outside of the active site in the apo- (+)-LS structure to a position close to the substrate and metal binding site in the (–)-LS structure. This residue could be important for stabilizing reaction intermediates, preventing water from entering the active site, or providing additional stability to metal coordination through a hydrogen bond with D488. Protein sequence alignment and

structural investigation for a broad subset of terpene synthases across all types (monoterpene, sesquiterpene, diterpene, plant, fungal, and bacterial) show that this residue is nearly 100% conserved (data not shown). Such invariance suggests that the residue would be required for catalytic activity, and indeed, mutation of this Tyr to Phe did decrease activity 50-fold. Clearly, the coordinated closure of the active site upon substrate and metal binding, and the involvement of Y565 in that transition, must be studied in greater detail.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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ABBREVIATIONS

GPP	geranyl diphosphate
FPP	farnesyl diphosphate
GGPP	geranylgeranyl diphosphate
LPP	linalyl diphosphate
CD	circular dichroism
GC-MS	gas chromatography–mass spectrometry
rmsd	root-mean-square deviation

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Figure 1.

Chemical mechanism proposed for the formation of limonene from geranyl diphosphate (GPP).^{6,11} The stereochemistry of intermediate species is not represented; however, the same mechanism is predicted to form either (+)-(4*R*)- or (-)-(4*S*)-limonene depending on the initial folding of GPP in the Michaelis complex and whether (3*R*)- or (3*S*)-LPP is formed as an intermediate.

Figure 2.

Identification of (+)-limonene as the major product and determination of enantioselectivity of the (+)-LS reaction. (A) Gas chromatogram for the product of an overnight reaction of GPP and (+)-LS showing the production of limonene, with smaller amounts of other monoterpenes. (B) Molar CD spectra for the enzymatic product from the reaction shown in panel A (black), a (+)-limonene standard (red), and a (-)-limonene standard (blue). The color code is the same for panels A and B.

Figure 3.

Michaelis-Menten plot for reaction of GPP with (+)-LS. This figure shows a plot of reaction velocity (nanomolar limonene produced per second; ordinate) vs GPP concentration (micromolar; abscissa). Each reaction mixture contained 20 nM (+)-LS, the indicated concentration of GPP substrate (1–200 μM), and 400 μM MnCl_2 . Reactions were performed as described in Experimental Procedures. The reaction for each concentration of GPP was performed in triplicate, where error bars represent the standard error of the mean. The data were fit to a rectangular hyperbola by nonlinear regression analysis with a K_M of 13.1 ± 0.6 μM and a k_{cat} of 0.186 ± 0.002 s^{-1} .

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Figure 5.
Superposition of the active site of apo-(+)-LS (blue) and FLPP-bound-(-)-LS (green; PDB entry 2ONG) structures.

Figure 6. Tertiary interactions important for stabilizing the H- α 1 helix region in the open [(+)-LS] and closed [(-)-LS] (PDB entry 2ONG) conformations of the enzyme. (A) Hydrogen bonds between the H- α 1 and I helices are shown as dotted lines in the open conformation of apo-(+)-LS. (B) The open conformation of apo-(+)-LS (blue) superposed with the closed conformation of FLPP-bound (-)-LS (green) reveals a hinge at P503.

Figure 7.

Similarity of amino acid residues in the active site binding pocket of apo-(+)-LS (blue) and FLPP-bound (-)-LS (green, PDB entry 2ONG) structures. The substrate analogue and J–K loop have been removed for the sake of clarity. Residues are numbered according to the (+)-LS sequence. In the case in which sequences diverge, the single-letter amino acid code following the forward slash corresponds to the (-)-LS structure.

Table 1**Crystallographic Data Collection and Refinement Statistics**

PDB entry	5UV0
Data Collection	
space group	$P4_12_12$
resolution range (Å)	20–2.3
highest-resolution shell (Å)	2.42–2.3
unit cell parameters (Å)	$a = b = 85.8$, $c = 216.4$
total no. of reflections	762563
no. of unique reflections	36820
completeness (%) ^a	99.8 (99.6)
$R_{\text{merge}}(\%)^a$	10.3 (116)
$I/\sigma(I)^a$	17.2 (2.1)
CC(1/2) (%) ^a	99.9 (71.6)
redundancy ^a	20.7 (12.6)
Refinement	
resolution range (Å)	20–2.3
no. of reflections used	36688
$R_{\text{cryst}}(\%)$	19.0
$R_{\text{free}}(\%)$	22.9
no. of protein atoms	4316
no. of water molecules	174
rmsd for bond lengths (Å)	0.007
rmsd for bond angles (deg)	0.8

^aValues for the highest-resolution shell are given in parentheses.