Pre-Steady-State Kinetic Analysis of the Trichodiene Synthase Reaction Pathway†

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ABSTRACT: The pre-steady-state kinetics of the trichodiene synthase reaction were investigated by rapid chemical quench methods. The single-turnover rate was found to be 3.5−3.8 s^{-1}, a rate 40 times faster than the steady-state catalytic rate ($k_{cat} = 0.09\ s^{-1}$) for trichodiene synthase-catalyzed conversion of farnesyl diphosphate (FPP) to trichodiene at 15 °C. In a multiturnover experiment, a burst phase ($k_b = 4.2\ s^{-1}$) corresponding to the accumulation of trichodiene on the surface of the enzyme was followed by a slower, steady-state release of products ($k_{lin} = 0.086\ s^{-1}$) which corresponds to k_{cat}. These results strongly suggest that the release of trichodiene from the enzyme active site is the rate-limiting step in the overall reaction, while the consumption of FPP is the step which limits chemical catalysis at the active site. Single-turnover experiments with trichodiene synthase mutant D101E, for which the steady-state rate constant k_{cat} is $^1/_3$ of that of wild type, revealed that the mutation actually depresses the rate of FPP consumption by a factor of 100. The deuterium isotope effect on the consumption of [1-^{2}H,1,2-^{14}C]FPP was found to be 1.11 ± 0.06. Single turnover reactions of [1,2-^{14}C]FPP catalyzed by trichodiene synthase were carried out at 4, 15, or 30 °C in an effort to provide direct observation of the proposed intermediate nerolidyl diphosphate (NPP). However, no NPP was detected, indicating that the conversion of NPP must be too fast to be observed within the detection limits of the assay. Taken together, these observations suggest that the isomerization of FPP to NPP is the step which limits the rate of chemical catalysis in the trichodiene synthase reaction pathway.

Trichodiene synthase is one member of a remarkable group of versatile catalysts which together are responsible for the formation of nearly 300 distinct sesquiterpene carbon skeletons (Croteau & Cane, 1985). Each synthase is capable of converting the universal acyclic precursor farnesyl diphosphate to a distinct sesquiterpene, while utilizing a common mechanism involving ionization of the allylic pyrophosphate ester followed by a precise sequence of intramolecular electrophilic addition reactions (Cane, 1985, 1990). A major determinant of the structure and stereochemistry of the ultimately formed sesquiterpene is believed to be the precise folding of the FPP[1] substrate at the enzyme active site.

Trichodiene synthase, which has been isolated from a variety of fungal sources (Evans & Hanson, 1976; Cane et al., 1981a,b; Hohn & Beremand, 1989a; Hohn & VanMiddlesworth, 1986), catalyzes the cyclization of *trans,trans*-farnesyl diphosphate (FPP, **1**) to trichodiene (**2**) (Cane, 1981, 1985, 1990). The enzyme has been extensively studied with respect to the mechanism, enzymology, and molecular genetics (Cane, 1990; Cane & Yang, 1994; Cane et al., 1995a,b). The cyclase from *Fusarium sporotrichioides*, a homodimer of a 45 kDa subunit, has been cloned (Hohn & Beremand, 1989b; Hohn & Plattner, 1989) and overexpressed in *Escherichia coli* (Cane et al., 1993). Extensive experiments with labeled substrates (Cane, 1981; Cane et al., 1985; Cane & Ha, 1988), as well as with substrate and intermediate analogs (Cane et al., 1990, 1992, 1995a; Cane & Yang, 1994), have provided strong support for the cyclization mechanism illustrated in Scheme 1 in which FPP, folded as shown, undergoes initial ionization and rearrangement to (3*R*)-nerolidyl diphosphate [(3*R*)-NPP, **3**]. Rotation around the 2,3-bond of **3** followed by reionization to generate a cisoid allylic cation−pyrophosphate anion pair allows cyclization to yield the bisabolyl cation (**4**). This isomerization−cyclization sequence is necessary to overcome the geometric barrier to direct cyclization of the *trans,trans*-farnesyl diphosphate precursor to the *cis*-cyclohexene-containing bisabolyl cation. Further cyclization of **4** followed by a 1,4-hydride shift is believed to give **5**, from which a consecutive pair of 1,2-methyl migrations and a final deprotonation result in the formation of trichodiene (**2**).

The intermediacy of (3*R*)-NPP has been supported by several lines of stereochemical and kinetic evidence (Cane et al., 1985, 1990; Cane & Ha, 1988; Cane & Yang, 1994). On the other hand, competition experiments between labeled samples of FPP and NPP have established that there is no interconversion of enzyme-free allylic diphosphate substrates and that (3*R*)-NPP is never released from the active site of the enzyme (Cane & Ha, 1988). Indeed, none of the proposed enzyme-bound intermediates have ever been directly observed in the course of cyclization.

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[1] Abbreviations: EDTA, ethylenediaminetetraacetic acid, disodium salt; FPP, farnesyl diphosphate; HPLC, high-pressure liquid chromatography; MeCN, acetonitrile; NPP, nerolidyl diphosphate; TD, trichodiene; WT, wild-type.

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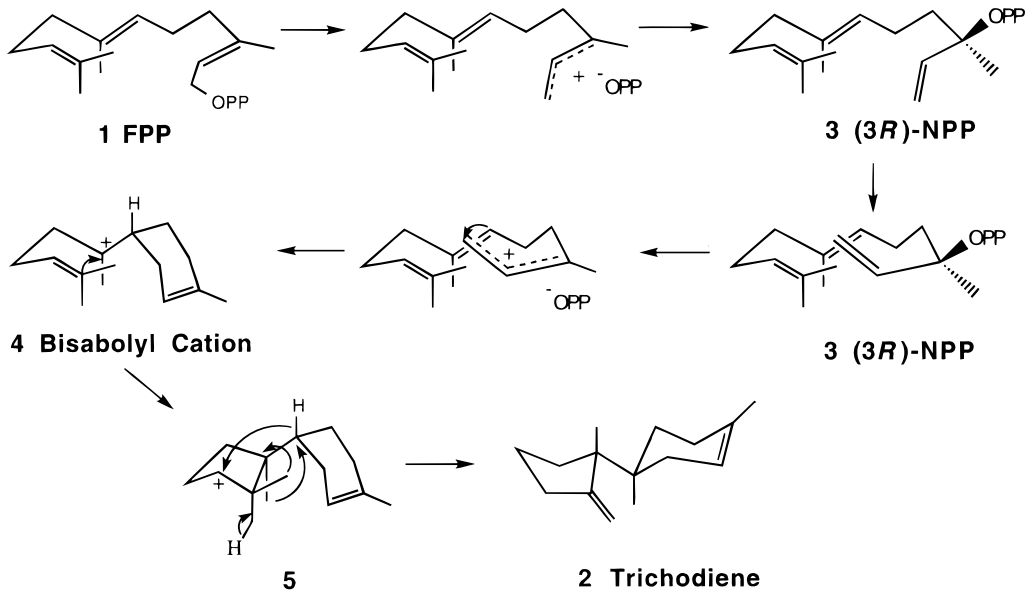
Scheme 1: Isomerization−Cyclization of FPP to Trichodiene



Rapid transient kinetic methods have provided a successful approach for the detection of enzyme intermediates (Anderson & Johnson, 1990a; Johnson, 1986, 1992). We now report the use of rapid chemical quench techniques in analyzing the pre-steady-state kinetics of the trichodiene synthase reaction and describe experiments designed to observe the proposed intermediate, NPP.

MATERIALS AND METHODS

Materials. Recombinant wild-type trichodiene synthase was purified from *E. coli* BL21(DE3)/pZW03 cells as previously described (Cane et al., 1995a,b). The D101E mutant of trichodiene synthase was purified as described from *E. coli* BL21(DE3)/*pQX01* cells (Cane et al., 1996a). Sources of reagents and chromatographic materials were also as previously described (Cane et al., 1995a,b). [1-^{3}H]FPP (41.7 and 78.7 μCi/μmol) was prepared by diluting [1-^{3}H]-FPP purchased from NEN DuPont with synthetic FPP, prepared as described (Cane &Yang, 1994). [1,2-^{14}C]FPP (50 and 55 μCi/μmol) and [1-^{2}H,1,2-^{14}C]FPP (55 μCi/μmol) were purchased from American Radiolabeled Chemicals, Inc. (ARC). (3*S*)-[1-*Z*]-*trans*-[1-^{3}H]Nerolidyl diphosphate (60 μCi/μmol, 6 $\times$ 10^4 dpm/μL) was synthesized as previously described (Cane & Ha, 1988). HPLC solvents were purchased from EM Science and were degassed by purging with He. All solvents for reactions were distilled prior to use. All reagents and buffer components used for enzyme assays and protein purification were of the highest quality commercially available.

General Methods. Methods for spectroscopic analysis, protein purification, and trichodiene synthase assays have been previously described (Cane et al., 1995a,b). Pre-steady-state measurements were made using a KinTek RFQ-3 Rapid-Chemical Quench apparatus (KinTek Instruments, State College, PA) equipped with a thermostatically controlled circulator (Johnson, 1986, 1992). Buffer T consisted of 10 mM Tris-HCl (pH 7.8), 5 mM $MgCl_2$, 15% (v/v) glycerol, and 5 mM β-mercaptoethanol. For all quench experiments, the substrate was prepared by diluting FPP with buffer T lacking glycerol and β-mercaptoethanol in HPLC-grade water. [1,2-^{14}C]FPP and [1-^{2}H,1,2-^{14}C]FPP as obtained from ARC were both found by HPLC to contain ca. 20% radiochemical impurities. Each individual radiochemical contaminant contributed 3−5% of the total radioactivity and was found to be chemically and enzymatically inert. FPP itself was completely consumed when incubated for extended quench times with trichodiene synthase, as determined by HPLC analysis. Analytical HPLC separations were performed on a Rainin HPLC system equipped with a automated gradient controller and a model UV-D II absorbance detector. NMR spectra were obtained on a Bruker AM 400 spectrometer at 400.134 MHz for ^{1}H and 162 MHz for ^{31}P. Radioactivity measurements were obtained on a Beckman LS 5801 liquid scintillation counter with 5 or 15 mL of Optifluor cocktail (Packard) and were automatically quench corrected.

Synthesis of (3RS)-trans-Nerolidyl Diphosphate. (±)-*trans*-Nerolidol (124 mg, 564 μmol, Aldrich) was stirred with (1,2-dibromo-1-phenylethyl)phosphonic acid (213 mg, 1.1 equiv) in 3 mL of dry CH_2Cl_2 in a flame-dried glass vial. The resulting cloudy suspension was clarified by addition of freshly distilled diisopropylethylamine (146 mg, 2 equiv). The reaction mixture was stirred at room temperature for 8 h. The solvent was removed by a gentle stream of nitrogen. Bis(triethylammonium) hydrogen phosphate (398 mg, 3.3 equiv), prepared as described (Cane et al., 1981), and freshly distilled CCl_3CN (500 μL) were added, followed by addition of dry acetonitrile (3 mL). The reaction mixture was stirred at room temperature overnight. Removal of the solvent was followed by addition of 50 mM $KHCO_3$ (1 mL) and extraction with ether (3 $\times$ 2 mL) to recover the starting alcohol. To the aqueous phase was added 30 μL of a solution of (3*S*)-[1-*Z*]-*trans*-[1-^{3}H]nerolidyl diphosphate (60 μCi/μmol, 6 $\times$ 10^4 dpm/μL) as an internal standard. The resulting mixture was loaded onto a DEAE-Sephadex A-25 anion exchange column (23 cm $\times$ 1.5 cm) which was equilibrated with 1 M $KHCO_3$ for 3 h at a flow rate of 0.4 mL/min and then equilibrated with 50 mM $KHCO_3$ for 9 h at the same flow rate. The column was then eluted with a linear gradient of 0.05 to 1 M $KHCO_3$. The fractions with the highest radioactivity were collected and lyophilized to give a white powder. The powder was then dissolved in 5

mL of deionized water (pH 8.5, adjusted by ammonium hydroxide). The resulting solution was loaded onto a polystyrene column (CHP-20P resin, Mitsubishi Chemical Industries, 10 cm × 2 cm) which had been washed with 100 mL of methanol and equilibrated with 200 mL of deionized water (pH 8.5). The column was washed with 60 mL of water (pH 8.5), followed by a linear gradient of 5 to 50% MeOH. Fractions were collected on the basis of the radioactivity and ^{31}P NMR. Upon removal of methanol, the solution was lyophilized to dryness to afford ca. 3 mg of white solid. ^{31}P NMR (D_2O, 162 MHz): −6.10 (1P, d, J = 21 Hz), −13.86 (1P, d, J = 21 Hz).

Steady-State Kinetics. Steady-state kinetic parameters were determined as previously described in buffer T (Cane, 1995a). Each series of assays was measured in duplicate using eight different concentrations of [1-^{3}H]FPP, ranging from 16 to 800 nM. Incubations were carried out for 10 min at 15 °C before quenching with 100 mM EDTA. [1-^{3}H]-Trichodiene generated from the incubation was mixed with 5 mL of Optifluor cocktail and quantitated by liquid scintillation counting. Data obtained from these experiments were analyzed by direct, nonlinear least-squares fitting to the Michaelis−Menten equation using KaleidaGraph simulation software (Synergy Software).

HPLC Analysis. (A) HPLC Separation of NPP, FPP, Farnesol, Nerolidol, and Trichodiene. HPLC separations were carried out by a modification of a previously published procedure (Poulter & Zhang, 1993). Separation of *trans*,*trans*-FPP and (3*RS*)-*trans*-NPP in buffer T was monitored at 214 and 230 nm upon elution from a BDS Hypersil C_{18} column (250 mm × 4.6 mm, 5 μm, Keystone Scientific, Bellefonte, PA) with a linear gradient of 5 to 100% MeCN in a 25 mM NH_4HCO_3 solution (pH 7.2, adjusted by CO_2) at a flow rate of 1 mL/min. Optimal resolution was achieved with a gradient slope of 1.6% MeCN/min, giving a difference in retention time of 1.76 min between NPP (t_R = 25.59 min) and FPP (t_R = 27.35 min). The same elution conditions were used to determine the retention times for farnesol (*cis*−*trans* isomers, t_R = 50.54−51.22 min), (3*RS*)-*trans*-nerolidol (t_R = 52.27 min), and trichodiene (t_R = 67.46 min).

(B) HPLC Analysis of Quenched Mixtures. The quenched incubation mixture was mixed with unlabeled (3*RS*)-*trans*-NPP and *trans*,*trans*-FPP as internal standards and analyzed by HPLC. The effluent from the column was monitored at 214 and 230 nm and then mixed with liquid scintillation cocktail (Ultima-Flo, Packard, or Mono-Flow V, National Diagnostics) at a flow rate of 3 mL/min. Radioactivity was monitored continuously by a Radiomatic 150 TR Flo-One radioactivity detector (Packard Instruments, Downers Grove, IL). The analysis system was automated by the use of an automatic sample injector (Waters 712B WISP, Milford, MA, or Rainin Instruments Dynamax, model AI-1A.) UV absorbance was monitored at 214 nm using a Rainin model UV-DII absorbance detector or a Waters 484 tunable absorbance detector.

Control Experiments. A number of control experiments were conducted to establish the chemical stability of substrates and intermediates under quenching conditions and the effectiveness of the chemical agent in terminating the enzymatic reaction.

(A) Stability of NPP and FPP under Quench Conditions. The stabilities of NPP and FPP with respect to the conditions of the quench reaction were examined using (3*S*)-[1-*Z*]-*trans*-[1-^{3}H]NPP (60 μCi/μmol) and [1,2-^{14}C]FPP (55 μCi/μmol). The radiolabeled compound in buffer T was treated with 0.6 N KOH under quench conditions in the absence of trichodiene synthase. The resulting solution was left at room temperature for 24−27 h. Analysis by HPLC showed that both FPP and NPP remained essentially intact and that the total radioactivity of each sample could be recovered.

(B) Effectiveness of 0.6 N KOH as a Quenching Reagent. In order to ensure that the base was completely quenching the enzyme reaction, control experiments were included with each experiment. These involved adding the enzyme to a premixed solution of 0.6 N KOH and the substrate and adding the substrate to a premixed solution of the enzyme and the base. No trichodiene was generated under either of these conditions.

(C) Mg^{2+}-Catalyzed Solvolysis of FPP. To carry out the quench experiments, FPP was premixed with $MgCl_2$ in buffer T lacking glycerol and stored at −20 °C before use. Examination of the Mg^{2+}-catalyzed solvolysis of FPP was included as a control. A solution of 21.88 μM [1,2-^{14}C]-FPP (55 μCi/μmol) in buffer T (no glycerol) was stored in a −20 °C freezer for 2.5 months. Hexane was used to extract any solvolyzed product from the FPP solution. No ^{14}C radioactivity was detected in the hexane extract.

(D) Stability of Trichodiene Synthase. Trichodiene synthase (800 μL of a 20.41 μM solution) was concentrated to 50−85 μM using a Microcon-30 apparatus (Amicon). The concentrate was stored at −78 °C and analyzed after several different periods of storage. Activity assay showed that the enzyme preparation retained full activity under these conditions for at least 1 month.

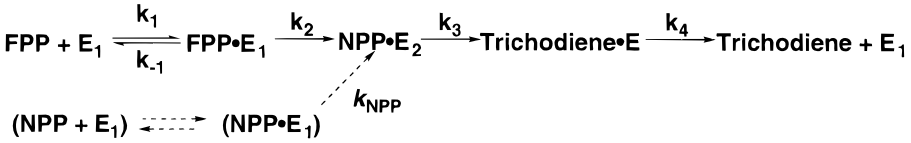
Rapid Quench Studies. The enzyme reaction was initiated by rapid mixing of the enzyme solution (15 μL) with the radiolabeled substrate (15 μL). To enhance the stability, trichodiene synthase in buffer T was kept cold by an ice/water circulator before loading into the sample loop of the rapid quench apparatus while the substrate in buffer T lacking glycerol and β-mercaptoethanol was maintained at room temperature during the experiments. In all cases, the concentrations of enzyme and substrate cited in the text are those after mixing and during the enzymatic reaction. The reaction was then quenched by mixing with 67 μL of 0.6 N KOH to give a final concentration of 0.4 N KOH.

For HPLC analysis of the quenched mixture, the quenched reaction solution was collected in a 1 mL Wheaton glass vial, vortexed, and immediately subjected to HPLC analysis. To obtain the pre-steady-state kinetic data, the quenched reaction solution was collected in a 1 mL Wheaton glass vial containing hexane (450 μL), vortexed, and immediately frozen in liquid nitrogen before analysis by hexane extraction. The hexane was included to prevent loss of trichodiene due to evaporation from the quenched mixture.

An aliquot of the radiolabeled substrate solution purchased from ARC was reduced to 5−10 μL by a gentle stream of argon. Buffer T (no glycerol) was then added to adjust the substrate concentration. In single-turnover experiments, 30−42 μM enzyme was reacted with 5−8 μM FPP (concentrations after mixing) at 4, 15, or 30 °C. The concentrations after mixing of the enzyme and the substrate in multiturnover experiments at 15 °C were 7.3 and 30 μM, respectively.

Deuterium Isotope Effect Experiments. Deuterium isotope effect experiments were carried out at 15 °C under single-turnover conditions. The same batch of the most active

Scheme 2: Kinetic Model for Conversion of FPP to Trichodiene



[a] KINSIM kinetic modeling software gave the following rate constants (see Materials and Methods): $k_1 \geq 1.2 \times 10^6$ M^{-1} s^{-1}; $k_{-1} = 0.1$ s^{-1}; $k_2 = 4$ s^{-1} ($k_{-2} = 0$ s^{-1}); $k_3 = 200$ s^{-1}; and $k_4 = 0.09$ s^{-1}. E_1 is the free enzyme, which can bind either FPP or exogenously added NPP, and E_2 is the form of the enzyme which is thought to catalyze cyclization of enzymatically generated (3*R*)-NPP. The dotted arrows indicate the proposed pathway for cyclization of exogenously added (3*R*)-NPP.

enzyme preparation was used for incubations with both [1,2-^{14}C]FPP and [1,2-^{14}C,1-^{2}H]FPP.

Pre-Steady-State Kinetics. Each vial containing a quench mixture and hexane was removed from storage at −80 °C; hexane (0.5 mL) was immediately added, and the quench mixture was thawed. Trichodiene and alcohols were extracted with hexane from the aqueous mixture. The hexane extract was then passed through a silica gel pipette column (2 cm) which was further washed with ether to flush out any alcohols. After extraction with hexane, the residual aqueous solution was collected and the inside of the vial was washed thoroughly with 0.2 N KOH. The aqueous solution, hexane extract, and ether wash were each mixed with 15 mL of scintillation cocktail separately in glass scintillation vials and subjected to liquid scintillation counting for 10 min.

Data Analysis. Raw disintegrations per minute data for the aqueous portion of the reaction product (corresponding to residual FPP) were corrected for the presence of unreactive radiochemical impurities present in the commercial samples of [1,2-^{14}C]- and [1-^{2}H,1,2-^{14}C]FPP. This background radioactivity was readily determined by incubation of labeled FPP for long quench times (6 s to 2 min), after which times all FPP had been consumed, as determined by HPLC, and measuring the residual ^{14}C activity of the aqueous phase. The actual amount of FPP remaining in the aqueous solution at each quench time was calculated by subtracting this constant background from the experimental disintegrations per minute. The accuracy of this method of data analysis was dependent on recovery of a reproducible volume of quenched solution or correcting for the actual recovered volume. In a like manner, the concentration of trichodiene was calculated from the hexane-extractable disintegrations per minute after correction for background. For single-turnover experiments, the data could also be analyzed by calculating the (background-corrected) fraction of the total activity corresponding to FPP remaining at each time point or the fraction of the total activity present as trichodiene at each time point.

Corrected data obtained by the methods above were fitted by nonlinear regression to the appropriate kinetic expression using KaleidaGraph. For example, for single-turnover experiments, the time course of consumption of substrate FPP was fit to the first-order rate equation (eq 1a), while the time course of the appearance of trichodiene was fit to eq 1b.

$$y = A_1 \exp(-k_{sFPP}t) + C_s \quad (1a)$$

$$y = A_2[1 - \exp(-k_{sTD}t)] \quad (1b)$$

where y is the corrected concentration of radioactive FPP or trichodiene, respectively, A_1 is the amplitude corresponding to the initial concentration of FPP (micromolar), A_2 is the maximum observed trichodiene concentration, k_{sFPP} is the exponential decay rate constant for consumption of FPP, k_{sTD} is the exponential rate constant for formation of trichodiene, t is the time in seconds, and C_s is the end point FPP concentration. For multiturnover experiments, the data for the residual FPP concentration were fit to the biphasic rate equation (eq 2)

$$y = A_3 \exp(-k_{bFPP}t) - L_{FPP}t + C_b \quad (2)$$

where y is the corrected concentration of residual FPP, A_3 is the observed burst amplitude (micromolar), k_{bFPP} is the exponential decay rate constant for the burst phase, L_{FPP} is the rate constant for the linear component of the reaction, and C_b is the end point of the single-exponential decay.

The KINSIM kinetic simulation program using numerical integration (Barshop et al., 1983), modified to allow the input of data from the rapid quench experiments as *x*,*y* pairs and to calculate the sum square errors in fitting the data (Anderson et al., 1988), was used to model the kinetic data presented in this report. The kinetic simulations were carried out using the model shown in Scheme 2 to provide a minimum estimate for k_3, the rate constant for conversion of NPP to trichodiene. Taking into account the mechanism illustrated in Scheme 1, k_3 itself is a complex rate constant covering all the steps between NPP and trichodiene summarized in Scheme 2. The KINSIM simulation used as the constraints the known K_m and k_{cat} values for the conversion of FPP to trichodiene by trichodiene synthase. The value for the rate constant for FPP binding, k_1, was set using the second-order catalytic efficiency constant (k_{cat}/K_m, 1.2×10^6 M^{-1} s^{-1}) as a lower limit for k_1, since $k_{cat}/K_m = [k_1k_2/(k_{-1} + k_2)]$. The rate of dissociation, k_{-1}, was set at 0.1 s^{-1} on the basis of k_1 and the K_m of 78 nM for FPP. The choice of these parameters did not significantly affect the value calculated by KINSIM for k_3, the rate of consumption of NPP. The value for the rate constant corresponding to conversion of FPP to NPP, k_2, was set at 4 s^{-1} on the basis of the values of k_{sFPP} and k_{bFPP} obtained from single-turnover and pre-steady-state burst experiments (see Results and Table 1). The reverse reaction, k_{-2}, is believed to be essentially negligible, as indicated earlier by competitive incubation experiments with FPP and NPP (Cane & Ha, 1998).[2] The lower limit for the value of k_3 was calculated by KINSIM

[2] Although there is no interconversion of free FPP and NPP (Cane & Ha, 1988), in principle, some reversal of the FPP to NPP conversion might be taking place on the enzyme, provided that the regenerated FPP never escapes the active site. If k_{-2} were >0, the effect would be reduction of the observed net rate constant for conversion of FPP to NPP [$k_2' = k_2k_3/(k_{-2} + k_3)$], resulting in an *underestimate* of k_3, the rate of conversion of NPP to trichodiene.

Table 1: Steady-State and Pre-Steady-State Kinetic Parameters for Trichodiene Synthase

	trichodiene synthase		D101E
parameter	15 °C	30 °C	30 °C
K_m (nM)	78	90[a]	124
k_{cat} (s^{-1})	0.09	0.32[a]	0.093
k_{sFPP} (s^{-1})	3.47 ± 0.22[b]	14.2 ± 4.7[b,c]	0.138 ± 0.016[b,c]
k_{sTD} (s^{-1})	3.49 ± 0.30[b]	15.3 ± 2.7[b,c]	0.153 ± 0.020[b,c]
k_{sFPP} (s^{-1})	3.60 ± 0.22[d]		
k_{sTD} (s^{-1})	3.81 ± 0.22[d]		
k_{bFPP} (s^{-1})	4.15 ± 0.27[d]		
k_{linFPP} (s^{-1})	0.086 ± 0.008[d]		

[a] Cane et al. (1995b). [b] Calculated from the relative fraction of FPP or trichodiene. [c] Data obtained by HPLC and in-line detection of radioactivity. [d] Calculated from corrected concentrations of FPP and trichodiene.

to account for the fact that NPP was not observed in an enzyme single-turnover experiment. The detection limits of the radioactive assay are approximately 0.7% (200 dpm of putative intermediate from a total of 30 000 dpm per assay) using HPLC analysis with a continous flow radioactivity detector. From this analysis, we predict that the rate of formation of trichodiene from NPP, k_3, must be at least 200 s^{-1} and also irreversible, such that k_{-2} is negligible. The rate of product dissociation from enzyme, k_4, is 0.09 s^{-1}, corresponding to the steady-state turnover rate, k_{cat}, as well as to the linear phase (k_{linFPP}) of the multiturnover experiment.

RESULTS

Single-Turnover Experiments. We have found that the conversion of substrate to product in a single enzyme turnover such that enzyme is in excess over substrate is an important diagnostic experiment for detecting enzyme intermediates (Anderson & Johnson, 1990a). Moreover, this type of experiment can also establish the fact that a putative intermediate is kinetically competent and along the reaction pathway (Anderson & Johnson, 1990b). In order to directly observe the formation of NPP as an intermediate in the trichodiene synthase reaction pathway and to establish the kinetic competence of NPP, the conversion of FPP to trichodiene was examined in a single enzyme turnover. This was accomplished by reacting [1,2-^{14}C]FPP with a 5-fold excess of trichodiene synthase at 15 °C in a rapid quench apparatus using transient kinetic techniques (Johnson, 1986, 1992, 1995). The reaction was quenched with aqueous KOH at time intervals ranging from 3 ms to several seconds. FPP and trichodiene were quantitated by liquid scintillation counting of the aqueous and hexane-extractable components. In a control experiment, trichodiene and any alcohols in the hexane extract were separated by a silica gel pipette column before liquid scintillation counting, which indicated negligible quantities of alcohols in the quench mixture. The time courses for FPP consumption and for trichodiene formation are shown in Figure 1. Under these conditions, there was no detectable intermediate, NPP, observed (see HPLC Analysis below). Analysis of the data, as described in Materials and Methods and summarized in Table 1, gave rate constants for FPP consumption and for trichodiene formation of 3.47−3.60 and 3.49−3.81 s^{-1}, respectively, in close agreement with each other. Significantly, no lag could be detected in the formation of trichodiene, even as early as 3 ms after mixing. Single-turnover experiments with varying

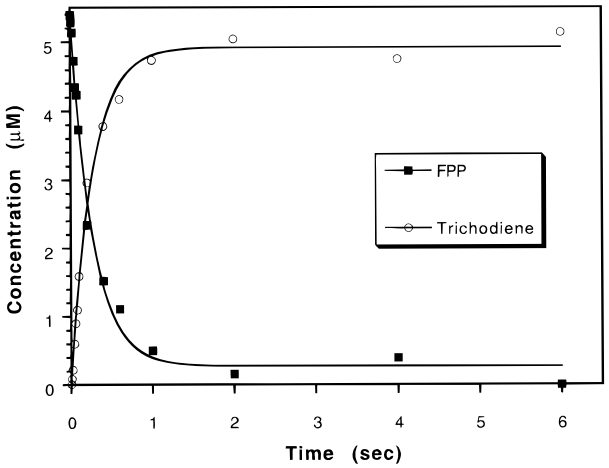


FIGURE 1: Time course for a single turnover of [1,2-^{14}C]FPP at the active site of trichodiene synthase at 15 °C. [1,2-^{14}C]FPP (5.41 μM) in buffer T lacking glycerol was rapidly mixed with enzyme (30 μM) (concentrations after mixing); after various times, the reaction was stopped by the addition of 0.6 N KOH. Data were analyzed using the background subtraction method as described in Materials and Methods. The data were fit to the appropriate first-order kinetic equation (eq 1a or 1b). Solid lines represent the calculated fit.

concentrations of trichodiene synthase at 15 °C gave the same rate constant, demonstrating that the binding of FPP to the enzyme active site was not rate-limiting. At 30 °C, the production of trichodiene reached its maximum within 500 ms under single-turnover conditions, while the consumption of FPP approached completion.

We have previously reported the steady-state kinetic behavior and product distribution of several trichodiene synthase mutants generated by site-directed mutagenesis of suspected active-site residues (Cane et al., 1995b, 1996a,b; Cane & Xue, 1996). One of the these mutants, trichodiene synthase D101E, was chosen for pre-steady-state, rapid chemical quench analysis. The latter enzyme exhibits a k_{cat} at 30 °C (0.093 s^{-1}) which is about $^1/_3$ of that of the wild-type trichodiene synthase under the same conditions (0.32 s^{-1}), with little change in the observed K_m [124 nM (D101E), compared to 90 nM (WT)]. Although the apparent decrease in k_{cat} is very modest, it was thought that slow product release might mask a more profound effect of the mutation on the rate of the actual chemical steps. If the chemical steps are perturbed by the mutation, this might result in a decrease in k_3 such that NPP would be observable. Indeed, in single-turnover experiments, the D101E mutant exhibited a 100-fold decrease in both the rate of FPP consumption (0.14 s^{-1}) and of sesquiterpene hydrocarbon formation (0.15 s^{-1}), compared to wild-type trichodiene synthase at the same temperature (Table 1). The formation of the intermediate, NPP, by the D101E mutant could not be detected however (see HPLC Analysis below).

Multiturnover Experiments. A multiturnover experiment was conducted with wild-type trichodiene synthase using a 4-fold excess of FPP over enzyme. The reaction was initiated at 15 °C by rapid mixing of 15 μL of [1,2-^{14}C]FPP (60 μM) in buffer T (no glycerol) with 15 μL of trichodiene synthase (14.6 μM) in buffer T (15% glycerol). The reaction was quenched with base at time intervals ranging from 3 ms to 8 s, and the concentrations of both FPP and trichodiene were determined. Rate constants were calculated from the consumption of FPP, which was observed to be biphasic,

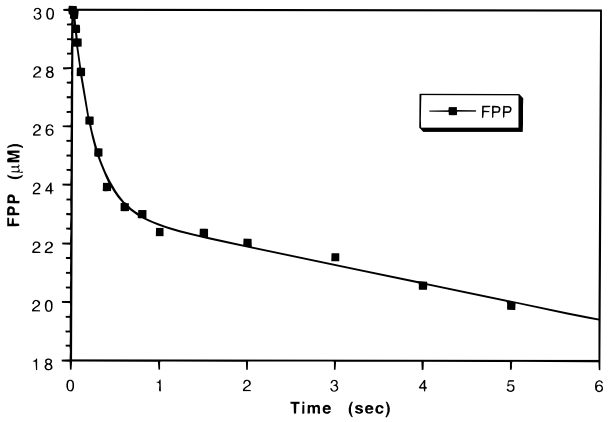


FIGURE 2: Time course of the pre-steady-state burst experiment at 15 °C. Trichodiene synthase (7.3 μM) was mixed with [1,2-^{14}C]-FPP (30 μM) (concentrations after mixing) in buffer T lacking glycerol in a rapid quench apparatus at 15 °C to start the reaction. The reaction was quenched by the addition of 0.6 N KOH and quantitated as described in Materials and Methods. Data were analyzed using the background subtraction method and fitted to the burst equation (eq 2) using starting parameters of 3.756 and 0.078 s^{-1} for the exponential and linear phases, respectively. The solid line represents the calculated fit.

characterized by a rapid, exponential phase followed by a slower linear phase (Figure 2). The first turnover took place at a rate of 4.15 s^{-1}, while subsequent turnovers occurred with a rate of 0.086 s^{-1}, in good agreement with both the single-turnover rate and the steady-state rate measured at 15 °C (Table 1, see below). The experimentally measured values for trichodiene concentration showed the same qualitative biphasic behavior. Due to unavoidable losses of the volatile, hydrophobic trichodiene product at longer quench intervals, however, as a result of evaporation and/or absorption to the tubing used in the rapid quench apparatus, the latter data were not precise enough to allow quantitative treatment. The amplitude of the burst in FPP consumption, and by extension trichodiene formation, corresponded to 93% of the expected enzyme active-site concentration. The pre-steady-state burst observed in this experiment clearly indicates that the rate-limiting step in the overall trichodiene synthase reaction occurs after the chemical step, the isomerization of FPP to NPP, and corresponds to release of the product from the active site.

Steady-State Kinetics. Steady-state kinetic measurements were conducted at 15 °C on wild-type trichodiene synthase in order to allow direct comparison with the pre-steady-state kinetic data at the same temperature. Varying amounts of [1-^{3}H]FPP were incubated with trichodiene synthase in buffer T to give 1−10% turnover during the 10 min assay period. Data were fitted directly to the Michaelis−Menten equation by nonlinear least-squares methods. The steady-state kinetic constants determined for trichodiene synthase-catalyzed conversion of farnesyl diphosphate (FPP) to trichodiene at 15 °C were a k_{cat} of 0.09 s^{-1} and a K_m of 78 nM. Previously reported steady-state kinetic data at 30 °C were a k_{cat} of 0.32 s^{-1} and a K_m of 90 nM (Cane et al., 1995b). The temperature change of 15 °C thus has an effect on k_{cat} of a factor of 3.6 but negligible effect on the observed K_m.

Deuterium Isotope Effect. To test whether the isomerization of FPP to NPP is indeed the rate-limiting chemical step in the trichodiene synthase reaction, we measured the deuterium isotope effect on the consumption of FPP under single-turnover conditions. Deuterium substitution at C-1 of FPP would be expected to exert a small, secondary isotope effect on the rate of FPP consumption if the isomerization were rate-limiting. In steady-state experiments, this isotope effect can only be detected by its influence on the measured k_{cat}/K_m, since the k_{cat} itself is dominated by the rate of product release. In principle, the isotope effect on the isomerization step can be measured by pre-steady-state methods. Single-turnover reactions using either [1,2-^{14}C]FPP or [1-^{2}H,1,2-^{14}C]FPP and a 4-fold excess of trichodiene synthase were carried out at 15 °C. The reactions were quenched with base at the same time intervals for each substrate, and the same batch of the most active enzyme preparation was used for both sets of experiments. The fractions of FPP consumed and of trichodiene formed were each fit to the appropriate exponential expression, revealing small but clear differences in rates for both deuterated and undeuterated substrates (Table 2), corresponding to measured secondary kinetic isotope effects of 1.1 on both FPP consumption and trichodiene formation.

Table 2: Secondary Deuterium Isotope Effects on Single-Turnover Rate Constants of Trichodiene Synthase

	$k_{(H-FPP)}$ (s^{-1})[a]	$k_{(D-FPP)}$ (s^{-1})[a]	k_H/k_D
k_{sFPP}	3.60 ± 0.14	3.26 ± 0.14	**1.11 ± 0.06**
k_{sTD}	3.50 ± 0.24	3.12 ± 0.21	**1.12 ± 0.11**

[a] Calculated from the relative fraction of FPP or trichodiene.

HPLC Analysis of Rapid Chemical Quench Reaction Mixtures. In the trichodiene synthase reaction, FPP is believed to undergo ionization and initial isomerization to the corresponding tertiary allylic pyrophosphate ester, nerolidyl diphosphate (NPP), which can then adopt the appropriate conformation for further cyclization to trichodiene, through a postulated series of carbocationic intermediates (Cane, 1990; Cane et al., 1992). The products of single-turnover reactions of FPP were analyzed to observe directly the proposed intermediate NPP, as well as any derived cyclized hydrocarbons or alcohols which might result from trapping of the cationic intermediates illustrated in Scheme 1. To this end, rapid quench experiments were performed under single-turnover conditions at 4, 15, and 30 °C using concentrations of [1,2-^{14}C]FPP and enzyme of 11 and 40 μM, respectively. Both the aqueous and hexane-extractable phases were analyzed by reverse-phase HPLC on a C_{18} column under conditions which had been shown to readily resolve *trans*,*trans*-FPP, *trans*-NPP, farnesol, (3*RS*)-*trans*-nerolidol, and trichodiene. Using a range of quench times from 3 ms to 5 min, however, we did not observe detectable levels of any intermediates, including NPP. Under these conditions, only residual substrate, FPP, and the final cyclization product, trichodiene, were observed.

KINSIM Kinetic Simulation of Reaction Rates. The failure to observe NPP as an intermediate prompted us to model the kinetic pathway using the KINSIM kinetic simulation program (Barshop et al.1983) as described in Materials and Methods and the rate information summarized in Table 2. This analysis, coupled with our radioactivity detection limits for observation of NPP (0.7% to the total radioactivity), suggests that the rate of breakdown of NPP to trichodiene (k_3 in Scheme 2) must be greater than 200 s^{-1} in order to account for the observed lack of accumulation of NPP, as revealed by the single-turnover rapid quench experiments.

DISCUSSION

Comparison of Pre-Steady-State and Steady-State Data. Data obtained from steady-state and pre-steady-state kinetic measurements are summarized in Table 1. At 15 °C, the rate-limiting chemical reaction, the consumption of FPP (k_{sFPP}), has a first-order rate constant that is about 40 times faster than the steady-state rate ($k_{cat} = 0.09$ s^{-1}). The latter rate was essentially identical to the rate of the release of product from the active site determined in the multiturnover experiments. At 30 °C, the observed steady-state rate increased by a factor of 3.6, while the single-turnover rates for FPP consumption and trichodiene formation increased by a comparable factors of 4.09 ± 1.38 and 4.39 ± 0.88. A single turnover of FPP at 30 °C was about 48 times faster than the steady-state turnover at this temperature, similar to the relative values of the rate constants observed at 15 °C.

These data indicate that release of trichodiene from the active site of the enzyme is the rate-limiting step in the overall reaction. Consistent with this conclusion were the results of the multiturnover experiment at 15 °C which showed that both the consumption of FPP and the production of trichodiene exhibited biphasic kinetics, with an initial burst with a rate constant of 4.15 s^{-1}, followed by a linear rate of 0.086 s^{-1}. The latter rate corresponded closely to the observed steady-state k_{cat} at the same temperature.

The observation that the rate-limiting release of trichodiene is 40 times slower than the slowest chemical step, isomerization of FPP, may be due to the interaction of the lipophilic product with hydrophobic active-site residues or the necessity of a slow change in protein conformation so as to allow release of product from the active site that must sequester the various cationic intermediates so as to protect them from premature quenching by water.[3]

The catalytic efficiency for the enzyme-catalyzed conversion of FPP to trichodiene, k_{cat}/K_m, is 1.2×10^6 s^{-1} M^{-1} at 15 °C and 3.6×10^7 s^{-1} M^{-1} at 30 °C. These rates, which are within 2 orders of magnitude of the diffusion-controlled rate, reflect a chemical step, and not the rate of binding of FPP, since the observed single-turnover rate constant was *independent* of protein concentration. The rapid chemical steps catalyzed by trichodiene synthase are masked by the slow release of the final product, trichodiene, which controls k_{cat}.[4]

The behavior of the trichodiene synthase D101E mutant is also of considerable interest. Aspartate 101 is part of an acidic motif, DDSKD, which appears to be involved in binding of the required divalent cation, Mg^{2+} (Cane et al., 1996a). Indeed, the DDXXD motif has been found in all known monoterpene, sesquiterpene, and diterpene synthase sequences, as well as in the mechanistically related FPP synthases (Cane et al., 1996a; Ashby & Edwards, 1990). The D101E mutant displays profound changes in product distribution, with five sesquiterpene hydrocarbons being produced in addition to the natural cyclization product trichodiene. The additional sesquiterpenes appear to be abortive products resulting from premature quenching of the normal cyclization intermediates. On the other hand, the D101E mutant exhibits only modest changes in the observed k_{cat} and K_m compared to those of wild-type trichodiene synthase (Table 1). We reasoned that the slow rate of product release (0.32 s^{-1}) for the wild-type trichodiene synthase might be masking more substantial effects of the mutation on the actual chemical steps in the conversion of FPP to trichodiene and other hydrocarbons. Indeed, pre-steady-state, rapid chemical quench analysis of the D101E mutant revealed that both the rate of FPP consumption and the initial rate of total sesquiterpene hydrocarbon formation were depressed by a factor of 100 compared to those of wild-type trichodiene synthase. Since the rate of consumption of FPP (k_{sFPP}) is the rate-determining chemical step, the observed k_{cat} is equal to $(k_{sFPP}k_{offTD})/(k_{sFPP} + k_{offTD})$, where k_{offTD} is the rate of product release (k_4). For wild-type trichodiene synthase, $k_{sFPP} \gg k_{offTD}$, and the observed k_{cat} is determined essentially by the product off rate (0.32 s^{-1}). Based on the observed k_{cat} (0.093 s^{-1}) of the D101E mutant, assuming no significant change in the rate of release of trichodiene, the slowest chemical step can be calculated to have a net rate constant of 0.13 s^{-1}, in excellent agreement with the measured rates of FPP consumption (k_{sFPP}) and sesquiterpene formation (k_{sTD}). The D101E mutation therefore has no *measurable* effect on the rate of consumption of NPP or any other downstream intermediate.

If the initial ionization and rearrangement of the pyrophosphate moiety of FPP is indeed the rate-determining chemical step, the substitution of deuterium at C-1 of FPP would be expected to exert a secondary deuterium isotope effect on the single-turnover rate. In fact, the measured secondary deuterium isotope effects of 1.1 measured for both substrate consumption and product formation, while close to the precision limits of the experimental method, are nonetheless on the expected order of magnitude (Croteau et al., 1987).

Attempts To Observe NPP by Rapid Chemical Quench. Several lines of evidence (Cane et al., 1985, 1990; Cane & Yang, 1994) have provided strong, albeit indirect, support for the intermediacy of (3*R*)-NPP in the conversion of *trans,trans*-FPP to trichodiene (Scheme 1). On the other hand, neither (3*R*)-NPP nor any derived intermediate has ever been directly detected in an incubation. Indeed, competition experiments have indicated that (3*R*)-NPP remains enzyme-bound (Cane & Ha, 1988). In the present study, attempts to observe NPP by rapid chemical quenching, even at temperatures of 4 °C and the shortest quench times of 3 ms, failed to reveal a detectable level of any intermediates derived from [1,2-^{14}C]FPP, nor could any lag be detected in the formation of trichodiene.

We have reported that the K_m for (3*R*)-NPP, the proposed intermediate, is essentially identical to that for *trans,trans*-FPP, the natural substrate for trichodiene synthase, while the k_{cat} and k_{cat}/K_m values for *trans,trans*-FPP are 1.67 times greater than those of exogenously added (3*R*)-NPP (Cane et al., 1995a). It has been proposed that the origin of this apparent anomaly is the possibility that the conformational state (E_1) of free trichodiene synthase that encounters the natural substrate FPP, or exogenously added (3*R*)-NPP, may be different from the conformation of enzyme (E_2) that normally binds and processes enzymatically generated (3*R*)-NPP (Scheme 2). The measured k_{cat}/K_m for (3*R*)-NPP (**3**)

[3] Similar observations are reported in the accompanying paper (Mathis et al., 1997) for the tobacco sesquiterpene synthase, epiaristolochene synthase, and a group of derived chimeric mutants.

[4] Whether the slow step, k_4, corresponds to actual product release, a slow protein conformational change followed by rapid release of product, or some combination of the two cannot of course be determined by these rapid chemical quench experiments.

may therefore include a slow conformational change of the trichodiene synthase subsequent to binding of free (3*R*)-NPP. Since product release has now been found to be rate-limiting for the overall reaction, in order for the binding of NPP and the consequent conformational change to have the observed effect of $^3/_5$ on the observed steady-state rate of trichodiene formation, the rate of this conformational change at 30 °C must be no faster than 0.48 s^{-1}, which is $1.5k_{off}$ for trichodiene, assuming that the rates of binding of NPP and FPP to free trichodiene synthase are identical. Notably, the *net rate* of consumption of exogenously added NPP is only slightly more than $^1/_8$ (3.6/0.48) of the rate of FPP consumption and *more than 400 times slower* than the rate of consumption ($k_3 = 200$ s^{-1}) of enzymatically generated NPP calculated by KINSIM kinetic simulation! In principle, it should be possible to test these predictions directly by carrying out single-turnover experiments with (3*R*)-NPP as the substrate.

CONCLUSIONS

Sesquiterpene synthases are responsible for the formation of nearly 300 distinct cyclic sesquiterpenes. In spite of the diversity of individual cyclization products, each of these enzymes uses a common substrate, *trans*,*trans*-FPP, and variations of a common cyclization mechanism to generate its characteristic product. The means by which each enzyme imposes a specific folding on the initially bound substrate and stabilizes the numerous cyclization intermediates are still obscure. The pre-steady-state kinetic analysis of trichodiene synthase described in this paper has shed new light on the transient kinetic behavior of a prototypical sesquiterpene synthase. Further studies with site-directed mutants, designed to detect enzyme-bound intermediates and to dissect the effects of individual mutations on both catalysis and binding, are in progress.

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