Trichodiene Synthase. Identification of Active Site Residues by Site-Directed Mutagenesis†

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ABSTRACT: Derivatization of 5,5′-dithiobis(2-nitrobenzoic acid)-treated trichodiene synthase with [*methyl*-^{14}C]methyl methanethiosulfonate and analysis of the derived tryptic peptides suggested the presence of two cysteine residues at the active site. The corresponding C146A and C190A mutants were constructed by site-directed mutagenesis. The C190A mutant displayed partial but significantly reduced activity, with a reduction in k_{cat}/K_m of 3000 compared to the wild-type trichodiene synthase, while the C146A mutant was essentially inactive. A hybrid trichodiene synthase, constructed from amino acids 1−309 of the *Fusarium sporotrichioides* enzyme and amino acids 310−383 of the *Gibberella pulicaris* cyclase, had steady state kinetic parameters nearly identical to those of the wild-type *F. sporotrichioides* enzyme. From this parent hybrid, a series of mutants was constructed by site-directed mutagenesis in which the amino acids in the base-rich region, 302−306 (DRRYR), were systematically modified. Three of these mutants were overexpressed and purified to homogeneity. The importance of Arg304 for catalysis was established by the observation that the R304K mutant showed a more than 25-fold increase in K_m, as well as a 200-fold reduction in k_{cat}. In addition, analysis of the incubation products of the R304K mutant by gas chromatography−mass spectrometry (GC−MS) indicated that farnesyl diphosphate was converted not only to trichodiene but to at least two additional $C_{15}H_{24}$ hydrocarbons, *m/e* 204. Replacement of the Tyr305 residue of trichodiene synthase with Phe had little effect on k_{cat}, while increasing the K_m by a factor of ca. 7−8. By contrast, the Y305T mutant exhibited an approximately 120-fold reduction in k_{cat} as well as an 80-fold increase in K_m. Moreover, the Y305T enzyme converted farnesyl diphosphate to an approximately equal mixture of trichodiene and an unidentified sesquiterpene hydrocarbon.

Trichodiene synthase (TS[1]) catalyzes the cyclization of farnesyl diphosphate (FPP, **1**) to trichodiene (**2**), the parent sesquiterpene hydrocarbon of the trichothecane family of antibiotics and mycotoxins (Cane et al., 1981). Although there are at least 200 individual sesquiterpene synthases, each responsible for the cyclization of FPP to a distinct sesquiterpene, trichodiene synthase is by far the most thoroughly characterized of these cyclases, with respect to mechanism, enzymology, and molecular genetics (Cane, 1990, 1995). Extensive mechanistic studies have lent strong support for the cyclization mechanism illustrated in Scheme 1 according to which FPP undergoes initial ionization and rearrangement to the tertiary allylic isomer, (3*R*)-nerolidyl diphosphate ((3*R*)-NPP, **3**), followed by a second ionization and electrophilic cyclization involving the central double bond (Cane et al., 1985, 1990, 1992; Cane & Ha, 1988). Further cyclization of the intermediate bisabolyl cation **4**, coupled with a series of hydride shifts and methyl migrations, ultimately leads to formation of the product trichodiene. In the preceding article in this issue, we described studies of the substrate specificity and inhibition of trichodiene synthase (Cane et al., 1995). We now report investigations designed to identify the amino acid residues that are present at the active site of trichodiene synthase and to clarify their role in binding and catalysis.

Trichodiene synthase has been isolated from a variety of fungal sources, including *Trichothecium roseum* (Evans & Hanson, 1976; Cane et al., 1981), *Fusarium sambucinum* (*Gibberella pulicaris*) (Hohn & Beremand, 1989a), and *Fusarium sporotrichioides* (Hohn & VanMiddlesworth, 1986). The *F. sporotrichioides* cyclase has been purified to homogeneity and found to be a homodimer of M_r 45 000 subunits, as determined by SDS−PAGE. The corresponding structural gene has been cloned (Hohn & Beremand, 1989b; Hohn & Plattner, 1989) and subsequently overexpressed (Cane et al., 1993) as 25−30% of soluble protein in *Escherichia coli*. Homologous TS genes have also been characterized from the trichothecene producers *G. pulicaris* (Hohn & Desjardins, 1992), *Myrrothecium roridum*, and *Gibberella zeae* (T. M. Hohn, unpublished results). Com-

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[1] Abbreviations: BGG, bovine γ-globulin; CD, circular dichroism; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); FPP, farnesyl diphosphate; IPTG, isopropyl β-D-thiogalactopyranoside; MMTS, methyl methanethiosulfonate; GC−MS, capillary gas chromatography−mass spectrometry; FPLC, fast protein liquid chromatography; LBA, Luria−Bertani ampicillin; NTB, 2-nitro-5-thiobenzoic acid; PCR, polymerase chain reaction; PMSF, phenylmethanesulfonyl fluoride; PP_i, inorganic pyrophosphate; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; TS, trichodiene synthase.

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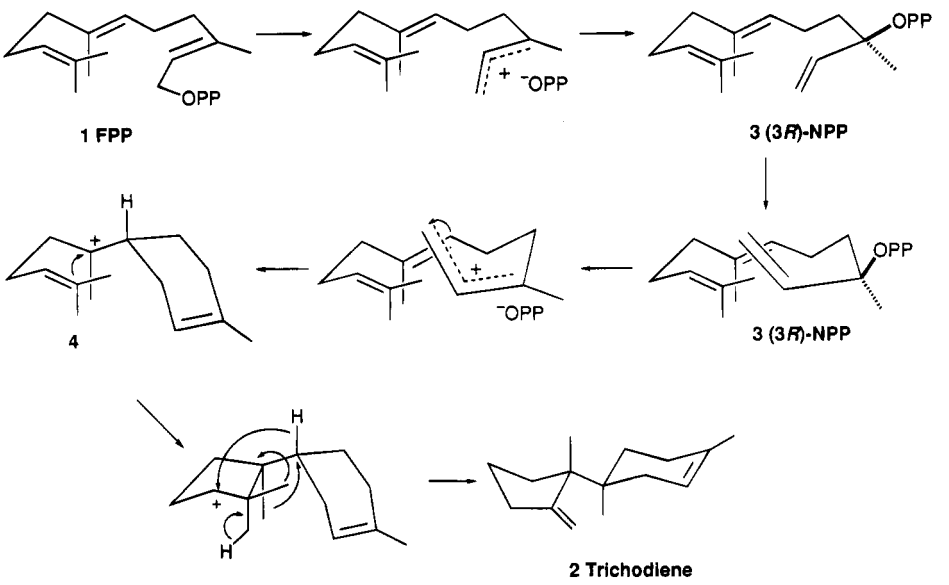
Scheme 1: Cyclization of FPP to Trichodiene



parison of the sequences of the *F. sporotrichioides* and *G. pulicaris* enzymes showed that they have 89% identity at the nucleotide level, including the presence of a 60-nucleotide intron, and 96% identity at the amino acid level. Nine additional amino acids were found in the C-terminal region of the *G. pulicaris* enzyme. On the other hand, none of the TS genes showed any significant sequence similarity to any other known genes or proteins.

In spite of the lack of overall sequence homology to other known proteins, the TS enzymes do contain two short consensus sequences of potential functional significance. The basic amino acid-rich sequences, DRRYR and DHRYR, are found in the *F. sporotrichioides* and *G. pulicaris* enzymes, respectively, and a closely related motif, LERYR, has been found in another sesquiterpene synthase, pentalenene synthase (Cane et al., 1994). Prenyl transferases such as FPP synthase share many mechanistic and enzymological similarities with the monoterpene and sesquiterpene synthases. It is therefore particularly interesting that photoaffinity labeling of avian liver FPP synthase with (*o*-azidophenyl)-ethyl pyrophosphate has been found to label a basic arginine residue in the sequence, EE**R**YK, implying that this side chain could be involved in binding of the pyrophosphate moiety (Brems et al., 1981). Sequencing of the rat and human fetal liver FPP synthases has revealed similar sequences, although the analogous site in the yeast enzyme is occupied by a basic lysine residue (Cane, 1990).

The aspartate-rich motif (I, L, or V)*X*DD*XX*D has been found in yeast hexaprenyl diphosphate synthase and in several FPP synthases as well (Ashby & Edwards, 1990; Ashby et al., 1992; Marrero et al., 1992). It has been proposed that the acidic aspartate residues are involved in substrate binding by chelation of the divalent metal ion (Ashby & Edwards, 1990). The homologous sequence has been found in TS from both *F. sporotrichioides* and *G. pulicaris* as well as other sesquiterpene synthases, including 5-*epi*aristolochene synthase from tobacco (*Nicotiana tabacum*) (Facchini & Chappell, 1992), aristolochene synthase from *Penicillium roquefortii* (Proctor & Hohn, 1992), and pentalenene synthase from *Streptomyces* UC5319 (Cane et al., 1994).

To date, nothing has been reported about the active site of any sesquiterpene synthase. We describe experiments using both amino acid specific reagents and site-directed mutagenesis, which identify a number of amino acid residues that apparently are important for catalysis of the trichodiene synthase reaction.

MATERIALS AND METHODS

Materials. The sources of reagents, [1-^{3}H]FPP, recombinant TS from *E. coli* BL21(DE3)/pZW03, and chromatographic materials were as described in the preceding paper (Cane et al., 1995). Supor modified polysulfone membrane (0.45 μm) was purchased from Gelman Science. Affi-gel Blue was purchased from Bio-Rad. MMTS, DTNB, iodoacetic acid, and iodoacetamide were purchased from Sigma. Stock solutions of DTNB (10 mM) were prepared in 0.1 M Tris Cl (pH 7.8). MMTS (10 mM) was dissolved in the appropriate buffer immediately before each experiment. [*methyl*-^{14}C]MMTS (0.06 mmCi/mmol) was prepared as described (Currier & Mautner, 1977) from potassium hydrogen sulfide (Johnson Matthey) and [^{14}C]methyl iodide (100 μCi, 8.7 mCi/mmol, Pathfinder Co.). All reagents and buffer components used for enzyme assays and protein purification were of the highest quality commercially available.

General Methods. General methods for spectroscopic analysis, protein purification and analysis, trichodiene synthase assay, and numerical analysis of the kinetic data were as described in the preceding paper (Cane et al., 1995). Buffer T consisted of 10 mM Tris-HCl (pH 7.8), 5 mM $MgCl_2$, 15% glycerol, and 5 mM β-mercaptoethanol. For sulfhydryl modification experiments, all incubations and assays were performed in buffer T lacking β-mercaptoethanol. Enzyme samples were desalted using a PD-10 gel filtration column to remove β-mercaptoethanol or $MgCl_2$, as needed. Ultraviolet and visible spectra were recorded on a Perkin-Elmer Lambda 3B UV/vis spectrophotometer or a Hewlett-Packard 8452A diode array spectrophotometer. GC−MS analyses of trichodiene and products of mutant and wild-type TS were performed on a Hewlett-Packard 5898 gas chromatograph−mass spectrometer. CD spectra of native and mutant proteins

were recorded on a Jasco J-700 spectrometer at Columbia University.

High-pressure fast protein liquid chromatography was performed with Waters Model 501 pumps and a Model 680 automated gradient controller with an in-line Model 440 absorbance detector or on a Pharmacia automated FPLC system. Restriction endonuclease digestions, DNA ligations, preparation and transformation of competent cells, plasmid minipreps, and other standard recombinant DNA manipulations were carried out according to published procedures (Sambrook et al., 1989; Cane et al., 1993).

Protein Concentration Assay. Routine protein concentrations were estimated by the method of Bradford with commercial reagents (Bio-Rad) using bovine γ-globulin as standard (Bradford, 1976). For more precise determination of protein concentration, the A_{280} was measured using an extinction coefficient of 86 988 M^{-1} cm^{-1}. The latter value was obtained by direct UV measurement of a sample of homogeneous TS in deionized water. Aliquots of this solution were then lyophilized to dryness, and the residue was weighed on a Cahn Gram Electrobalance Model 1501. The extinction coefficient was calculated on the basis of M_D 43 999 per monomeric subunit. Comparison of the protein concentrations determined by direct UV assay with the results of the Bio-Rad assay indicated that the latter method overestimated protein concentrations by a factor of 5.5. The accuracy of the A_{280} assay was further confirmed by cysteine titration experiments using DTNB (Habeeb, 1972).

Purification of Trichodiene Synthase from E. coli BL21-(DE3)/pZW03. Trichodiene synthase was purified to homogeneity by a modification of the previously published procedure (Cane et al., 1993). This procedure was also used to purify TS mutants to homogeneity. In cases in which mutants had little or no activity, the purification was monitored by SDS−PAGE. Seed cultures (10 mL) of *E. coli* BL21(DE3)/pZW03 were used to inoculate LBA medium (3 × 650 mL). The cultures were grown at 37 °C on a shaker (250 rpm) until an OD_{600} of 1.2−1.4 was reached. IPTG (1 M, 0.65 mL) was added to each culture to a final concentration of 1 mM to induce the synthesis of TS, and the cultures were incubated at 30 °C on a shaker (250 rpm) for 3.5 h. Cultures were harvested by centrifugation (8000*g*, 15 min). The cells were suspended in 30 mL of lysis buffer (buffer T supplemented with 0.1 mM PMSF) (Cane et al., 1993) and recentrifuged (8,000*g*, 15 min) to remove any remaining LBA medium. Lysis buffer was added at 3 mL/g of wet cells and the pellet was resuspended. After 8 μL of 50 mM PMSF and 80 μL of lysozyme (10 mg/mL) were added per gram of *E. coli*, the resulting suspension was stirred for 20 min on ice. Triton X-100 (10%, v/v) was added to a final concentration of 0.5% (v/v) and the mixture was stirred for another 30 min. The lysate was then frozen at −78 °C and thawed at 30 °C. DNase I (20 μg/g of *E. coli*) was added to the lysate, and the mixture was stirred at room temperature until it was no longer viscous (about 1 h). Occasionally, brief sonication (3 min, 40% duty cycle, output 5, flat tip) was also used to reduce the viscosity. The resulting lysate was then centrifuged at 150000*g* (4 °C) for 65 min to remove cell debris. The supernatant was either purified immediately or stored at −78 °C.

(a) Ammonium Sulfate Fractionation. After diluting the cell homogenate with two volumes of lysis buffer, the solution was brought to 45% ammonium sulfate saturation by the slow addition of solid, powdered ammonium sulfate (25.8 g/100 mL) with stirring on ice. After 40 min, the resulting cloudy solution was centrifuged at 100000*g* (4 °C) for 40 min. The volume of 45% supernatant was measured and brought to 65% saturation by the slow addition of ammonium sulfate (12.3 g/100 mL). The solution was stirred for 40 min and then centrifuged at 100000*g* (4 °C) for 50 min. The precipitate was dissolved in buffer T (4 mL).

Table 1: Purification of Trichodiene Synthase from *E. coli* BL21(DE3)/pZW03[a]

step	total protein (mg)[b]	total activity (units)[c]	specific activity (units/mg)	recovery (%)
cell lysate	600	7854	13	
45−65% $(NH_4)_2SO_4$ ppt	260	6447	25	82
Q-Sepharose	29	5237	180	66
blue dye	19	3482	183	44
Superose 12	11	2193	199	27

[a] Data are based on the protein from 1 L of culture. [b] Bio-Rad protein assay with BGG as standard. [c] One unit of enzyme activity is defined as nanomoles of trichodiene/minute under the assay conditions described in the Materials and Methods Section.

(b) Anion Exchange Chromatography. The 45−65% ammonium sulfate precipitate dissolved in buffer T was filtered on a Supor modified polysulfone membrane to remove contaminating lipids. The filtrate was diluted by adding buffer T (100 mL) and loaded onto a Q-Sepharose column (2.5 × 13 cm) equilibrated with buffer T at a flow rate 1 mL/min. The column was washed with 2 bed volumes of starting buffer (100 mM KCl in buffer T) followed by a linear gradient of 100−350 mM KCl in buffer T (total of 400 mL), and 5 mL fractions were collected. Each fraction was assayed for protein concentration and enzyme activity. The fractions with the highest specific activity were collected. When the mutants were purified, the fractions were also routinely monitored by SDS−PAGE.

(c) Affinity Chromatography. The enzyme solution from the Q-Sepharose column was passed through an Affi-blue column (1.5 × 1 cm) by gravity. The eluent was loaded onto the column again, and the second eluent was concentrated to 0.5 mL using a YM-30 membrane (Amicon) and a Centricon-30.

(d) Gel Filtration Chromatography. The concentrated enzyme solution from the blue dye column was applied to a Superose-12 column (1.5 × 43 cm) equilibrated with buffer T. The column was eluted with buffer T at a flow rate of 0.35 mL/min. Fractions (1 mL each) were collected after the void volume (15 mL). Each fraction was assayed for protein concentration and enzyme activity. Fractions with the highest specific activity were analyzed by SDS−PAGE, and the highest purity fractions were used for further study. The purification of wild-type TS is summarized in Table 1.

Inactivation Studies. The inactivation of TS by the sulfhydryl-modifying reagents MMTS, DTNB, iodoacetic acid, and iodoacetamide was investigated. Samples that contained 0.5−1.0 μM enzyme in a volume of 0.2 mL were incubated at room temperature with the reagent under study. For protection studies, the required amount of FPP or PP_i was included in the incubation. $MgCl_2$ was excluded from the buffer in some incubations. At intervals, 5−10 μL aliquots were removed from the incubation mixture and

added to the assay mixture (0.5 mL, β-mercaptoethanol excluded) to assay for remaining TS activity. A control, which contained no sulfhydryl reagents, was run at each time point, and all inactivation rates were relative to this control.

Cysteine Determinations. The total number of cysteine residues was measured by a modification of the method of Habeeb (1972). The enzyme (0.1−2.0 nmol) was added to a cuvette containing 1.0 mM DTNB in 4.8 M guanidine hydrochloride in a final volume of 1.0 mL, and the absorbance at 412 nm was recorded. The reaction was complete within 15 min. The number of moles of nitrothiobenzoate anion released was calculated using the reported extinction coefficient of 13 600 M^{-1} cm^{-1}. The number of accessible cysteine residues in the native enzyme was also measured in the absence of guanidine-hydrochloride using an incubation time of 2 h. Protein concentrations were determined by A_{280} using the extinction coefficient of 86 988 M^{-1} cm^{-1} or by Bio-Rad protein assay as described, and the results were corrected by dividing by 5.5.

Titration of Trichodiene Synthase with [^{14}C]MMTS. Samples containing approximately 1 nmol of enzyme or DTNB-modified enzyme in a volume of 1.5 mL of buffer T without mercaptoethanol were incubated at room temperature with 2.4 μmol of [^{14}C]MMTS for 1 h. The incubation mixture was desalted on a PD-10 column to remove unreacted [^{14}C]MMTS. A portion of the desalted sample (50 μL) was used to measure the protein concentration, which was determined by the Bio-Rad protein assay and corrected by dividing the result by 5.5. The total radioactivity was determined by scintillation counting. The DTNB-modified enzyme was prepared by incubating the enzyme with DTNB at room temperature for 2 h. The NTB anion released from the reaction and the unreacted DTNB were removed using a PD-10 column.

Trypsin Digest of MMTS-Treated Trichodiene Synthase. Trichodiene synthase (2.5 mg) in a volume of 2.5 mL of buffer T without mercaptoethanol was incubated at room temperature with 2 mM DTNB for 2 h. The reaction mixture was passed through a PD-10 column, and the protein solution was divided into two 2 mL portions. To one sample were added 2 μmol of sodium PP_i and 2.4 μmol of [^{14}C]MMTS in 20 μL of ethanol. To the other sample was added 2.4 μmol of [^{14}C]MMTS. After 2 h of incubation at room temperature, unreacted [^{14}C]MMTS was removed using a PD-10 column equilibrated in 0.1 M NH_4HCO_3, pH 8.1. The samples were digested at 37 °C with trypsin (2%, w/w) for 12 h. The trypsin digestion was quenched by freezing the reaction mixture at −78 °C. The frozen digest was concentrated *in vacuo* to reduce the volume to 0.2 mL for HPLC injection.

Initial fractionation of the tryptic peptides was performed on a C-18 reverse phase column (μ-Bondapak, Waters, 1.2 × 23.5 cm). An 80 mL linear gradient was run from 100% solvent A (0.1% TFA) to 80% solvent B (acetonitrile/water, 9:1, v/v, 0.1% TFA) at 1.0 mL/min. After 80 min, the eluent was changed to 100% solvent B. The eluent was monitored at 214 nm, and fractions of 1 mL were collected. Aliquots (0.5 mL) from each fraction were counted for ^{14}C radioactivity (5 min). The radioactive fraction from the μ-Bondapak column was further purified on a Vydac C-18 peptide/protein reverse phase column (0.6 × 22 cm). The radioactive fraction was concentrated to 0.05 mL before injection. An 80 mL linear gradient was run from 80% solvent A to 80% solvent B with a flow rate of 1.0 mL/min. After 80 min, the eluent was changed to 100% solvent B. Fractions (1 mL each) were collected and monitored at 214 nm. Aliquots (0.5 mL) from each fraction were counted for ^{14}C radioactivity (5 min). The isolated radioactive peptides were characterized by automated gas phase amino acid sequence analysis and matrix-assisted laser desorption mass spectrometry by Dr. William Lane at the Harvard Microchemistry Facility.

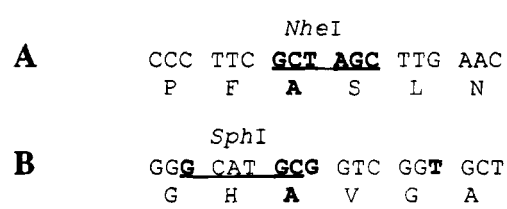


FIGURE 1: Nucleotide sequence changes in the trichodiene synthase coding sequences present in pTF02 (A) and pTF01 (B). Nucleotide changes are indicated in boldface type, and the restriction sites introduced by PCR are underlined. The translation of the nucleotide sequences is also shown, and the Ala residues substituted for Cys146 (pTF02) and Cys190 (pTF01) are indicated in boldface type.

Plasmids for Site-Directed Mutagenesis of Amino Acids 146 and 190. The Cys residues at amino acid (aa) positions 146 and 190 were changed to Ala using a modification of the inverse PCR method (Street et al., 1991). The PCR primers were designed so that the intended nucleotide (nt) changes for the Cys to Ala substitutions were incorporated into a unique restriction site that could be used for plasmid ligation. Primers 1F (5′-GTAGCTAGCTTGAACCTTATTCGCAGC-3′) and 1R (5′-GATGCTAGCGAAGGGACCAAAGTGCCG-3′), which contain *Nhe*I sites, were used for the position 146 substitution, while primers 2F (5′-TCGAGCATGCGGTCGGTGCTTCTCTATGGC-3′) and 2R (5′-ACGAGCATGCCCCAAGCCGTTCATAC-3′), which contain *Sph*I sites, were used for the position 190 substitution. PCR reactions were performed using these primer pairs and pZW03 (Street et al., 1991) as the template. Following PCR, the products were recovered using Geneclean (BIO101) and digested with either *Nhe*I (1F and 1R) or *Sph*I (2F and 2R). The restriction digests were separated on a 1% SeaPlaque (FMC BioProducts, Rockland, ME) gel and the plasmid-sized bands were excised. Ligation reactions containing the digested plasmids were used to transform competent XL1 Blue cells (Stratagene, La Jolla, CA). The resulting plasmids designated pTF01 (2F and 2R) and pTF02 (1F and 1R) were then introduced into BL21(DE3) by electroporation using a Cell-Porator (Bio-Rad, Hercules, CA). Electroporation competent cells were prepared according to the manufacturer's instructions for *E. coli*. DNA sequence analysis confirmed that only the intended sequence changes (Figure 1) had been introduced into the TS coding regions present in pTF01 and pTF02.

Characterization of Products from Trichodiene Synthase Mutants by GC−MS. In a typical experiment, the crude cell lysate from *E. coli* BL21(DE3)/pTF01 (C190A) (50 μL) was incubated with 12 μM FPP (71.7 Ci/mol) at 30 °C for 1 h. The hydrocarbon product was extracted with distilled pentane and purified on a silica gel column (3 cm) packed in a Pasteur pipet overlaid with sodium sulfate (0.5 cm). Identical incubations were performed with the cell lysate from *E. coli* BL21(DE3)/pZW03 (wild type) and with a preparation of purified wild-type trichodiene synthase. The extracts of each incubation were concentrated *in vacuo* at 0 °C, and the

Table 2: Nucleotide Changes in the Hybrid Trichodiene Synthase Coding Sequences

plasmid	restriction sites used for fragment insertion	nucleotide substitutions in the sequence corresponding to amino acids 302−306[a]
pTS91-1	*Eco*RV−*Kpn*I	GATCGCAGGTACCGC
pTS102-7	*Bsp*MI(filled-in)−*Kpn*I	**C**ACCGCAGGTACCGC
pTS104-6	*Bsp*MI(filled-in)−*Kpn*I	**G**A**G**CGCAGGTACCGC
pTS90-1	*Pvu*I−*Bgl*II	GA**T**A**T**CAGGTACCGC
pTS92-7	*Eco*RV−*Kpn*I	GAT**GAG**AGGTACCGC
pTS93-7	*Eco*RV−*Kpn*I	GATC**G**C**AA**GTACCGC
pTS94-2	*Eco*RV−*Kpn*I	GATCGC**GA**GTACCGC
pTS98-1	*Eco*RV−*Bgl*II	GATCGCA**G**GA**C**CCGC
pTS99-6	*Eco*RV−*Bgl*II	GATCGCAGG**C**A**G**CGC
pTS100-10	*Eco*RV−*Bgl*II	GATCGCAGG**T**T**C**CGC
pTS103-1	*Kpn*I−*Bgl*II(filled-in)	GATCGCAGGTA**C**A**AG**
pTS101-1	*Kpn*I−*Bgl*II(filled-in)	GATCGCAGGTAC**GAG**
pTS97-9	*Pvu*I−*Bgl*II, *Kpn*I−*Bgl*II(filled-in)	GAT**AT**CAGGTAC**GAG**

[a] Nucleotide substitutions are indicated in boldface type with underlining.

concentrates (1−3 μL) were analyzed by GC−MS. The observed spectra were compared with that of authentic trichodiene. All three samples consisted of a single component with identical retention times (6.6 min) and mass spectra, confirming that the enzymatically generated product was trichodiene. Analogous experiments were also carried out with the TS91-1 (fusion protein), TS93-7 (R304K), TS100-10 (Y305F), and TS98-1 (Y305T) mutants.

Plasmids for Site-Directed Mutagenesis of aa 302−306. To facilitate the substitution of synthetic DNA fragments specifying changes in aa 302−306, a hybrid TS coding sequence was constructed. The plasmid pGP58-1 (Hohn & Desjardins, 1992) was digested with *Bgl*II and *Kpn*I, and the resulting fragment containing nt 927−1149 of the coding region from the *G. pulicaris* TS gene and the plasmid pTZ18U (Pharmacia, Piscataway, NJ) was recovered using Geneclean (BIO101, La Jolla, CA). This fragment was ligated with a *Kpn*I−*Pvu*I fragment from pTS37-4Δ(470-529) (Hohn & Plattner, 1989), containing nt 1−906 of the TS coding sequence from *F. sporotrichioides* and a synthetic DNA fragment with *Pvu*I−*Bgl*II compatible ends. Plasmid pTS37-4Δ(470-529) carried a copy of the TS coding region from which the 60-nt intron had been specifically deleted, while the synthetic DNA fragment was constructed by hybridizing two complementary oligonucleotides following phosphorylation of the 5′-nucleotides (Hohn & Plattner, 1989). The sequence of the synthetic DNA fragment corresponded to nt 905−929 of the TS coding sequence from *F. sporotrichioides*, except that nt 907−908 were changed from CG to AT in order to create an *Eco*RV site in the hybrid. Change of nt 907 and 908 from CG to AT also results in an amino acid change at position 303 (Arg to Ile). Following the transformation of *E. coli* strain AG-1 (Stratagene, La Jolla, CA), a plasmid designated pTS77-2 was identified that contained nt 1−929 of the TS coding sequence from *F. sporotrichioides* and nt 930−1149 from the *G. pulicaris* TS. The hybrid TS sequence in pTS77-2 lacked the *Pvu*I site starting at nt 903 of the *F. sporotrichioides* sequence and contained an *Eco*RV site starting at nt 905. To express the TS hybrid in *E. coli*, it was first cloned into the expression vector pKK223-3 (Pharmacia). This was accomplished by digesting pTS77-2 with *Bam*HI, treating it with mung bean exonuclease to generate blunt ends, and then digesting it with *Pst*I. The fragment carrying the TS sequence was isolated and cloned into pKK223-3 that had been digested with *Eco*RI, blunt-ended by treatment with mung bean exonuclease, and digested with *Pst*I. The resulting plasmid, called pTS90-1, was used as the starting point for further plasmid constructions. Amino acid changes were introduced using specifically designed synthetic DNA fragments capable of insertion into the *Bsp*MI, *Eco*RV, *Kpn*I, and *Bgl*II sites (Table 2 and Figure 2). Restriction enzyme and DNA sequence analysis of each plasmid confirmed that the intended sequence changes had been introduced into the TS coding regions.

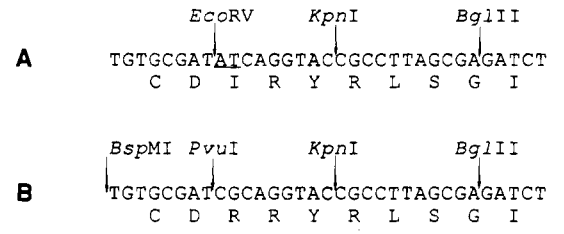


FIGURE 2: Translated sequence and restriction sites in the 900−930-nt region of hybrid TS coding sequences: (A) sequence from pTS90-1; (B) sequence from pTS91-1. Restriction enzyme cut sites are indicated by the arrows. The underlined sequence in (A) represents nt changes introduced to create the unique *Eco*RV site.

Trichodiene Synthase Activity in Transformants Carrying Plasmids Derived from pKK223-3. Cultures carrying plasmids derived from pKK223-3 were grown and induced with IPTG as previously described (Hohn & Plattner, 1989). Induced cultures were harvested by centrifugation, resuspended in buffer T, and then disrupted by sonication as described in Hohn and Plattner (1989). A portion of the cell homogenate was centrifuged in a microcentrifuge tube for 5 min at 12000*g* and the supernatant was recovered. Supernatant proteins were separated by SDS−PAGE, and the resulting polyacrylamide gels were stained with Coomassie Brilliant Blue R-250 (Sigma, St. Louis, MO) to estimate protein concentrations and compare protein profiles. Analysis of the stained gels revealed no significant differences in the 12000*g* supernatants with respect to either the amounts of protein or the protein banding pattern. To confirm the presence of TS polypeptide in the 12000*g* supernatants, they were submitted to immunoblot analysis using specific polyclonal antiserum directed against TS as described (Hohn & Beremand, 1989a). An intense band that comigrated with purified TS was observed in samples from all of the 12000*g* supernatants except the pKK233-3 control. Comparison of the TS bands indicated similar amounts (±20%) of TS polypeptide in all of the 12000*g* supernatants from cultures carrying TS coding sequences in pKK223-3. TS activity was determined as described (Hohn & Plattner, 1989).

Overexpression of Mutant Trichodiene Synthase Constructs. To overexpress a selected number of the altered TS coding sequences, *Nco*I−*Sac*I fragments (839 bp) from the pKK223-3-derived plasmids were inserted into the *Nco*I−*Sac*I sites of the TS coding sequence present in pZW03 (Cane et al., 1993). The plasmids were initially transformed into the *E. coli* strain XL1 Blue (Stratagene) and then moved to BL21(DE3) for overexpression. Transformation of BL21-(DE3) with pZW03-derived plasmids was accomplished by electroporation.

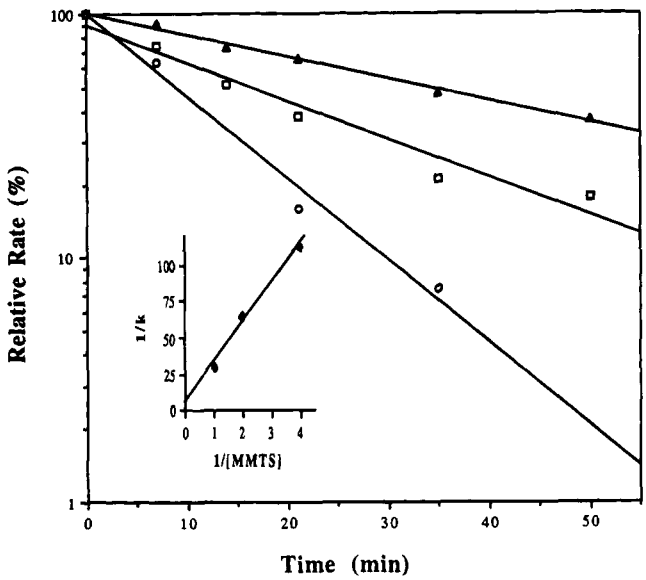


FIGURE 3: Kinetics of inactivation of trichodiene synthase by MMTS. Enzyme (0.8 μM) was incubated at room temperature with the following concentrations of MMTS: 0.25 (▲), 0.5 (□), and 1.0 mM (○) . Total incubation volume was 0.2 mL in buffer T without β-mercaptoethanol. At the indicated time intervals, 5 μL aliquots were removed and assayed for activity using the standard assay described under Materials and Methods. Plot of log(% residual activity) versus time. (Inset) Plot of $1/k_{app}$ versus 1/[MMTS].

DNA Sequencing. DNA sequencing of the 850−1000-nt region of the TS coding sequence in pKK233-3-derived plasmids was performed with a Sequenase version 2.0 kit purchased from U.S. Biochemical Corp. (Cleveland, OH). Sequencing reactions were labeled with [α-^{35}S]ATP. DNA sequencing of the entire TS coding sequence in pTF01 and pTF02 employed the Taq DyeDeoxy terminator cycle sequencing kit (Applied Biosystems, Foster City, CA). Sequencing reactions were analyzed using an Applied Biosystems Model 373A automated DNA sequencer.

RESULTS

Chemical Modification by Sulfhydryl-Directed Reagents. Several monoterpene synthases are known to be sensitive to sulfhydryl-directed reagents. Preliminary experiments demonstrated that MMTS inactivated trichodiene synthase in a time dependent fashion. Other cysteine modification reagents, such as DTNB, iodoacetic acid, and iodoacetamide, were not effective in inactivating the enzyme. When TS was treated with increasing concentrations of MMTS, the rate of inactivation increased. The inactivation was essentially irreversible since enzyme activity was not recovered by adding β-mercaptoethanol to the incubation mixture. After correction for the significant background of non-inhibitor dependent enzyme inactivation, a plot of log(percent residual activity) against time was linear, indicating that inactivation obeyed pseudo-first-order kinetics (Figure 3). A plot of $1/k_{app}$(inact) versus 1/[MMTS] was linear and gave an apparent K_i of about 4.60 mM and a maximum rate of inactivation, k_{inact}, of approximately 0.17 min^{-1} (Figure 3, inset). Increasing concentrations of FPP afforded protection against inactivation by MMTS, consistent with an active site-directed inactivation process.

Cysteine Determination. The DNA sequence of TS indicates that there are 7 cysteine residues per subunit of the homodimer. Enzyme samples were denatured with 4.8 M guanidinium chloride and assayed for total cysteine by titration with DTNB. As expected, there were 6.9 cysteine residues/mol of subunit. Assay of native TS with DTNB showed 5.9 titratable cysteine residues/mol of subunit. The reaction was slow and took 2 h for completion. This value indicates that there are no disulfide bonds in the structure, a conclusion supported by the observation that TS has maximum catalytic activity in the presence of β-mercaptoethanol. The result also indicates that an average of 1 cysteine residue per subunit is not modified by DTNB. DTNB-treated enzyme was not significantly inactivated beyond the competing background loss of activity, suggesting that the unmodified cysteine residue(s) might be at the active site.

To investigate this possibility further, MMTS was used to derivatize enzyme that had been preincubated with DTNB for 65 min. The DTNB-treated enzyme slowly lost activity over 2 h, but the addition of MMTS greatly accelerated the inactivation of trichodiene synthase (data not shown). This suggested that an internal cysteine residue, which had not been derivatized by DTNB, was modified by MMTS, resulting in enzyme inactivation.

Identification of Active Site Cysteine Residues. In order to identify the apparent active site cysteine, trichodiene synthase was incubated with [^{14}C]MMTS. When trichodiene synthase was incubated with [^{14}C]MMTS, there were 6.9 titratable cysteine residues/mol of subunit. On the other hand, when DTNB-modified enzyme was treated with [^{14}C]-MMTS, an average of 0.6 cysteine residue/mol of subunit was found to be labeled.

Since the active site cysteine residue appeared to react only with MMTS, an attempt was made to selectively label this residue by using DTNB to block cysteine residues that were not at the active site. DTNB-treated trichodiene synthase was reacted with [^{14}C]MMTS, both in the presence and absence of PP_i. The results of the two incubations were compared in order to detect differential labeling of tryptic peptides. The HPLC profiles from the separation of the tryptic digest of trichodiene synthase modified with [^{14}C]-MMTS either in the presence or absence of PP_i were almost identical in appearance (data not shown). Two peptides, eluting at 56 and 58 min, were found to have 3−5 times higher ^{14}C activity than the corresponding peaks obtained from the digestion of trichodiene synthase that had been incubated with MMTS in the presence of PP_i. Both of these peptides were rechromatographed, and a single radioactive peptide component was obtained from each. For each peptide, the first five amino acid residues were sequenced, giving the sequences ^{184}MNGLG and ^{141}HFGPF for peptides eluting at 56 and 58 min in the first chromatogram, respectively. Both peptides were analyzed by matrix-assisted laser desorption mass spectrometry. The molecular weight of each tryptic peptide including the modified cysteine residue was calculated, and the corresponding peak was assigned within an error range of ±0.25% of the observed weight. The first peptide (1394.0 ± 3.49) corresponded to ^{184}MNGLGHCVGASLW^{196}, which would have a calculated molecular weight of 1390.75 including CysSS^{14}CH$_3$. The unusual site of cleavage may be the result of contaminating chymotrypsin activity in the trypsin. The second tryptic peptide (1453.8 ± 3.6) was ^{141}HFGPFCSLNLIR^{152}, with a calculated molecular weight of 1449.8 including CysSS^{14}-CH$_3$. These latter results unexpectedly indicated that both Cys190 and Cys146 had been labeled with [^{14}C]MMTS.

Table 3: Relative Activities of Crude Extracts of Trichodiene Synthase Mutants Constructed by Site-Directed Mutagenesis in Amino Acid Residues 302−306[a]

trichodiene synthase mutants	302−306 region	trichodiene synthase activity (%)[b]
pTS91-1[Fs(1−309)/ Gp(310−383)]	DRRYR	100
pTS102-7(D302N)	NRRYR	93.9
pTS104-6(D302E)	ERRYR	92.5
pTS90-1(R302I)	DIRYR	100
pTS92-7(R303E)	DERYR	100
pTS93-7(R304K)	DRKYR	2.5
pTS94-2(R304E)	DREYR	0.1
pTS100-10(Y305F)	DRRFR	85.5
pTS98-1(Y305T)	DRRTR	0.2
pTS99-6(Y305Q)	DRRQR	0.1
pTS103-1(R306K)	DRRYK	94.3
pTS101-1(R306E)	DRRYE	14.3
pTS97-9(R303L & R306E)	DLRYE	3.2

[a] Hybrid trichodiene synthases expressed using pKK223-3. [b] Trichodiene synthase activity as a percent of pTS91-1 TS activity.

Table 4: Kinetic Parameters for Mutant Trichodiene Synthases

trichodiene synthase	K_m (nM)	k_{cat}[a] (s^{-1})	k_{cat}/K_m (s^{-1} M^{-1})
wild type	68.2	0.32	4.7×10^6
C190A	91.1	1.44×10^{-4}	1.6×10^3
C146A	nd[b]	$\sim 10^{-6}$	
fusion protein [Fs(1−309)/ Gp(310−383)]	54.3	0.23	4.2×10^6
R304K	1500	1.44×10^{-3}	9.6×10^2
Y305F	433	0.20	4.6×10^5
Y305T	4500	1.96×10^{-3}	4.4×10^2

[a] Per subunit of homodimer. [b] Not determined.

Characterization of Mutants C190A and C146A. To investigate the functional roles of Cys190 and Cys146 in enzyme catalysis, mutant strains of *E. coli* BL21(DE3) carrying pTF01 (C190A) and pTF02 (C146A) were constructed by site-directed mutagenesis. Each TS mutant was purified to homogeneity, and the steady state kinetic parameters, K_m and k_{cat}, were determined (Table 4). The CD spectra of each of the mutant proteins showed negligible differences when compared with that of native enzyme (data not shown.)

The k_{cat} for the C190A mutant was reduced by about 2000-fold relative to that of the wild-type enzyme, whereas the K_m was increased by about 1.3-fold. The cyclization product from mutant C190A was compared by GC−MS to an authentic sample of trichodiene, as well as to the product from wild-type enzyme. All three samples had identical GC retention times and mass spectra, confirming that the product from mutant C190A was indeed trichodiene. The specific activity of cell lysate from C146A was measured only in crude cell extracts.

Analysis of the Basic Amino Acid-Rich Domain of Trichodiene Synthase. To examine the functional significance of the basic amino acid-rich domain of TS, a series of mutants carrying amino acid substitutions at residues 302−306 was constructed (Tables 2 and 3). To facilitate the insertion of synthetic DNA fragments specifying the desired changes in aa 302−306, a hybrid TS coding sequence was first constructed using aa 1−309 of the *F. sporotrichioides* protein and aa 310−383 of the closely related *G. pulicaris* enzyme and carrying a CG to AT change in nt 907−908 of

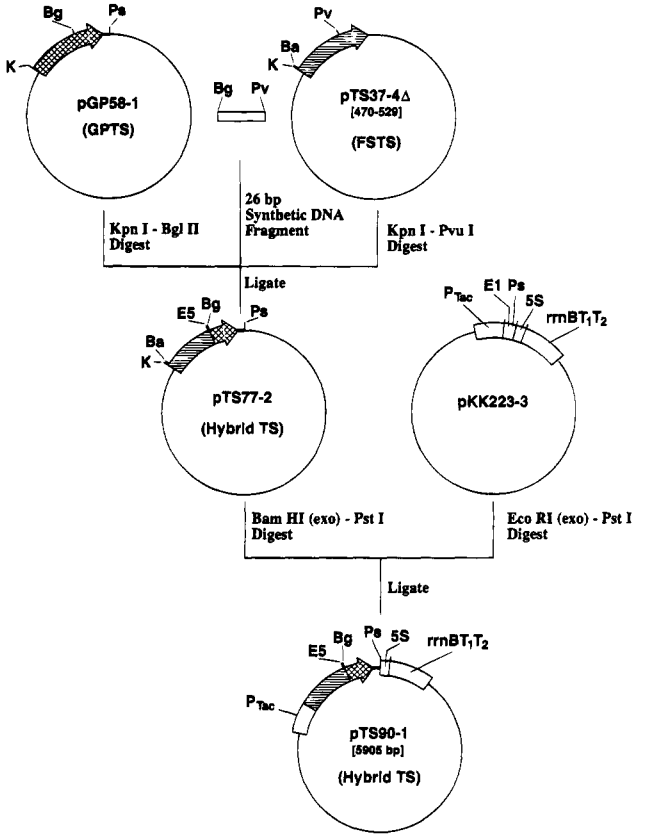


FIGURE 4: Diagrammatic representation of the cloning strategy used to construct pTS90-1. The trichodiene synthase gene is shown as an arrow. Abbreviations: GPTS, trichodiene synthase from *G. pulicaris*; FSTS, trichodiene synthase from *F. sporotrichioides*; exo, mung bean exonuclease treatment; P_{tac}, *E. coli* trp-lac promoter; 5S, 3′-end of *E. coli* 5S ribosomal RNA; $rrnBT_1T_2$, *E. coli* ribosomal RNA transcriptional termination sequences; Ba, *Bam*HI; Bg, *Bgl*II; E1, *Eco*RI; E5, *Eco*RV; K, *Kpn*I; Ps, *Pst*I; Pv, *Pvu*I.

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GPTS - YEKVKGQKTEDAEKFCKFYEQAANVGAVSPSEWAYPPIAQ -350
FSTS -      EE     Q                          V   -350

GPTS - LANIRTKDVKDLKDVKDLKEIQKPLLSSIELVE -383
FSTS -   V S    --------- V  F          -374
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FIGURE 5: Sequence differences between the portion of the hybrid trichodiene synthase contributed by *G. pulicaris* (GPTS) and the *F. sporotrichioides* trichodiene synthase (FSTS). Dashes indicate that no corresponding amino acids are present in FSTS.

the *F. sporotrichioides* coding sequence. The latter change resulted in the loss of a *Pvu*I site starting at nt 903 of the *F. sporotrichioides* sequence and the introduction of an *Eco*RV site starting at nt 905, as well as the substitution of Arg303 by Ile (Figure 2). The hybrid mutant was expressed in *E. coli* by subcloning into the expression vector pKK223-3. The resulting plasmid, pTS90-1, containing the newly introduced *Eco*RV restriction site, served as the starting point for the generation of all other mutants in the aa 302−306 range by insertion of synthetic DNA fragments (Tables 2 and 3) into appropriate restriction sites. The cloning strategy is described in the Materials and Methods section and summarized in Figure 4. Replacement of Ile303 with the wild-type amino acid Arg (pTS91-1) generated a fusion protein that appeared to be kinetically very similar to the wild-type *F. sporotrichioides* enzyme. Figure 2 compares the sequences of pTS90-1 and pTS91-1 in the nt 900−930 region, while Figure 5 summarizes the differences between the portions of the hybrid TS contributed by the *G. pulicaris* and *F.*

sporotrichioides enzymes. The fusion protein served as the comparison standard for the 12 mutants carrying substitutions in aa 302−306, which were each assayed as crude extracts. Western blots confirmed that each construct produced similar levels of TS protein.

Four of these mutant enzymes, the "wild-type" fusion protein TS91-1, as well as the R304K (TS93-7), Y305F (TS100-10), and Y305T (TS98-1) mutants, were selected for further study. Each of the mutant genes was subcloned into the previously described T7-based high-level expression system, and the resulting proteins were each purified to homogeneity, their steady state kinetic parameters were determined, and the products of incubation with FPP were analyzed. CD spectra of the fusion protein, as well as of the R304K and Y305F mutants, were each identical to that of native TS (data not shown; the Y305T mutant was not analyzed by CD). The *F. sporotrichioides*/*G. pulicaris* fusion protein had essentially unchanged K_m and k_{cat} values compared to those for the wild-type enzyme *F. sporotrichioides* synthase (Table 4). GC−MS analysis confirmed that the product of cyclization of FPP by TS91-1 was exclusively trichodiene.

When Arg304 in this construct was substituted with another basic residue, Lys, k_{cat} for TS93-7 was reduced by approximately 200-fold relative to the wild type, while K_m increased by approximately 22-fold, resulting in a decrease in k_{cat}/K_m of over 4000-fold. GC−MS examination of the pentane extracts resulting from the incubation of FPP with TS93-7 revealed that the product was a mixture of trichodiene (t_R = 6.72 min) and three unidentified sesquiterpene hydrocarbons, parent *m/e* 204, designated A (t_R = 6.54 min), B (6.59 min), and C (6.78 min), in an approximate ratio of 4(A):2(B):1(2):1(C). The structures of these anomalous cyclization products are currently under investigation. No alcoholic products or additional hydrocarbons were found in the ethereal extract of this same incubation mixture. When Phe was substituted for Tyr305 (TS100-10), k_{cat} was nearly the same as wild type ($^2/_3$) but there was 6-fold increase in K_m, resulting in a net 10-fold decrease in the k_{cat}/K_m value. The major cyclization product was again trichodiene, accompanied by <5% hydrocarbon A. By contrast, replacement of Tyr305 with Thr in TS98-1 not only resulted in an 80-fold increase in K_m, as well as a more than 120-fold decrease in k_{cat}, but the product was a ca. 1:1 mixture of trichodiene and the $C_{15}H_{24}$ product A. The fact that A was indeed derived from FPP was confirmed by the observation that the incubation of [1-$^2H_2$]FPP with TS98-1 shifted the parent peaks for both A and trichodiene to *m/e* 206.[2]

DISCUSSION

Identification of Active Site Cysteine Residues. Sulfhydryl groups are thought to be at or near the active site of several monoterpene cyclases (Rajaonarivony et al., 1992). All monoterpene cyclases have similar mechanistic features, and a sulfhydryl group at the active site may have a similar function in all of these enzymes. However, the mechanistic role of the sulfhydryl group in these enzymes has not been elucidated, nor have sequence homologies between these cyclases been revealed.

Trichodiene synthase was inactivated by reaction with MMTS. However, other thiol-directed reagents such as DTNB, iodoacetamide, and iodoacetic acid were poor inhibitors of the enzyme. Trichodiene synthase has seven cysteine residues in a subunit, and there has been no indication of the presence of disulfide bonds. In fact, all enzyme preparations and activity assays have been performed in the presence of 5 mM β-mercaptoethanol, and maximum enzymatic activity is shown under reducing conditions. The absence of disulfide bonds has been demonstrated by DTNB titration of the seven cysteine residues in each subunit when denatured by guanidinium chloride under nonreducing conditions. When free cysteine residues were titrated with DTNB, different types of sulfhydryl groups were evident. Of the six thiols that were modified with DTNB, five were derivatized after 1 h, while the sixth reacted more slowly.

The inactivation studies and titrations indicated that MMTS had reacted with at least one active site cysteine residue in trichodiene synthase. Since DTNB apparently modified an average of six cysteine residues, an attempt was made to selectively label the active site thiol with [^{14}C]-MMTS. In fact, two cysteine residues, Cys190 and Cys146, were found to be labeled. This result would appear to be inconsistent with the cysteine modification studies, which imply only one essential thiol. In fact, it is likely that the value of six cysteine residues modified by DTNB after 2 h of incubation represented a weighted average in which some species had greater or fewer than six blocked cysteines. Site-directed mutagenesis studies definitively showed that both cysteine residues that were labeled by MMTS were required for trichodiene synthase activity. Comparison of the CD spectra of each of the cysteine mutants revealed no detectable changes in protein secondary structure associated with the mutations. For the C190A mutant, there was a decrease in k_{cat} by 2000-fold, while K_m was increased by only 30% compared to that of the wild type. The product from this mutant was shown to be trichodiene by GC−MS comparison with an authentic sample. It appears that Cys190 contributes little to binding of the substrate or intermediates, but may participate in catalysis.

Identification of Amino Acids Involved in Substrate Binding. Earlier studies with photoaffinity labels indicated that an arginine-rich region of avian liver farnesyl diphosphate synthase may be involved in the binding of the pyrophosphate moiety of the substrate (Brems et al., 1981). Curiously, however, Joly and Edwards (1993) subsequently found that replacement of this "active site arginine-[192]" with lysine in the rat FPP synthase resulted in essentially insignificant changes in V_{max} or in the K_m for either substrate, isopentenyl diphosphate or dimethylallyl diphosphate. Therefore, it is all the more intriguing that changes in the corresponding DRRYR region of trichodiene synthase can have a considerable effect on both the steady state rate of product formation and even the nature of the products themselves. The results reported here thus suggest that the arginine-rich region of TS, amino acids 304−306 (RYR), may play an important role in both binding and catalysis. Only 2% activity was observed when Arg304 was substituted with Lys, while only 0.1% of the activity remained when this residue was replaced with Glu. Interestingly, replacement of Tyr305 with the nonpolar aromatic Phe reduced activity by only a small

[2] In our original manuscript, we had reported only the characterization of the cyclization product of the C190A mutant. We are grateful to a referee for suggesting that product analyses be carried out on all mutants.

amount, but substitution by nonaromatic Thr or Gln resulted in a significant reduction in activity. On the basis of CD spectra, neither the R304K (TS93-7) nor the Y305F (TS98-1) mutants had undergone any detectable alteration in protein secondary structure compared to the wild-type enzyme.

The substitution of Lys for Arg304 produced large changes in both K_m and k_{cat}, resulting in a 4000-fold reduction in catalytic efficiency, as measured by the k_{cat}/K_m value of TS93-7. This substitution, although a conservative change with respect to charge, could result in significant changes in interaction with the substrate, since the three nitrogen atoms in the δ-guanidino group of Arg, each of which can form a hydrogen bond, were exchanged for the one nitrogen atom of the ϵ-amino group of Lys. The amino group of Lys, with an intrinsic pK_a value of 11.1, is also a potent nucleophile in its nonionized form. The guanidino group of Arg not only is more basic, with a pK_a of 12, but the positive charge is distributed over the entire group as a result of resonance. The protonated form is unreactive, and only a very small fraction is present in its nonionized form at pH 7.8. Most interestingly, GC–MS analysis of the cyclization products revealed that the formation of trichodiene was accompanied by the formation of three new and as yet unidentified sesquiterpene hydrocarbon products (*m/e* 204: $C_{15}H_{24}$). Although incubation of substrate analogs with wild-type trichodiene synthase has been reported to result in the formation of abortive cyclization products (Cane et al., 1990; Cane & Yang, 1994), this is the first demonstrated example of the generation of anomalous products using a mutant terpenoid cyclase of any class. Since the precise folding of the substrate FPP and derived intermediates is believed to be a major determinant of the structure of the products eventually formed, the appearance of additional products presumably is the result of active site changes that affect substrate folding or intermediate stabilization. We are currently investigating the structures of these anomalous sesquiterpene products.

Phenylalanine is nearly isosteric with tyrosine, but lacks the polar phenolic hydroxyl group. The K_m of the Phe305 mutant, TS100-10, was increased by only 6-fold compared to that of the wild-type TS, with essentially no change in k_{cat}. The phenolic hydroxyl group of Tyr often participates in hydrogen bonding (Gerlt, 1987), and the loss of this interaction in the nearly isosteric phenylalanine residue may account for the small changes observed in K_m. Analysis of the cyclization products indicated that the major product was, as expected, trichodiene, accompanied by at most 5% of an *m/e* 204 component, which appeared to be identical in GC retention time and MS with hydrocarbon A produced by TS93-7.

Finally, substitution of Tyr305 with Thr led to a major loss in activity in mutant TS98-1, affecting both k_{cat} and K_m and resulting in a net 10^4 decrease in k_{cat}/K_m. It was also found that cyclization of FPP resulted in the formation of a mixture of sesquiterpene hydrocarbon products consisting of equal proportions of trichodiene and hydrocarbon A. These latter results may be due to the intrinsic importance of Tyr305 for trichodiene synthase catalysis, or they may be an indirect effect due to the improper alignment of Arg304 as a consequence of the loss of favorable stacking interactions with the adjacent aromatic amino acid side chain. Resolution of this issue will have to await the results of collaborative crystallographic studies now in progress.

Further experimentation will be required to confirm that amino acid residues 305 and 306 are part of the pyrophosphate binding site of TS and to distinguish effects on binding from those on catalysis. The actual functional roles of the two Cys residues that have been implicated in catalysis of the trichodiene synthase reaction remain obscure, and the significance of the aspartate-rich domains of trichodiene synthase and other terpenoid cyclases remains to be explored.

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