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Crystal Structure of Albaflavenone Monooxygenase Containing a Moonlighting Terpene Synthase Active Site*[S]

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Albaflavenone synthase (CYP170A1) is a monooxygenase catalyzing the final two steps in the biosynthesis of this antibiotic in the soil bacterium, *Streptomyces coelicolor* A3(2). Interestingly, CYP170A1 shows no stereo selection forming equal amounts of two albaflavenol epimers, each of which is oxidized in turn to albaflavenone. To explore the structural basis of the reaction mechanism, we have studied the crystal structures of both ligand-free CYP170A1 (2.6 Å) and complex of endogenous substrate (epi-isozizaene) with CYP170A1 (3.3 Å). The structure of the complex suggests that the proximal epi-isozizaene molecules may bind to the heme iron in two orientations. In addition, much to our surprise, we have found that albaflavenone synthase also has a second, completely distinct catalytic activity corresponding to the synthesis of farnesene isomers from farnesyl diphosphate. Within the cytochrome P450 α-helical domain both the primary sequence and x-ray structure indicate the presence of a novel terpene synthase active site that is moonlighting on the P450 structure. This includes signature sequences for divalent cation binding and an α-helical barrel. This barrel is unusual because it consists of only four helices rather than six found in all other terpene synthases. Mutagenesis establishes that this barrel is essential for the terpene synthase activity of CYP170A1 but not for the monooxygenase activity. This is the first bifunctional P450 discovered to have another active site moonlighting on it and the first time a terpene synthase active site is found moonlighting on another protein.

Cytochrome P450 monooxygenases (CYP or P450)[2] are members of the most structurally diverse and functionally versatile superfamily of heme-containing enzymes with more than 10,000 known genes distributed among all biological kingdoms. CYP proteins have extremely diverse primary sequences and are grouped into different families when they have less than 40% amino acid sequence identity. Only the *trans*-cysteine ligand coordinating with the heme iron atom is conserved among all P450s (1). CYP enzymes are involved both in endogenous biosynthetic functions and in the metabolism of xenobiotic chemicals (2) and catalyze the oxidation of a wide variety of substrates with both regiospecificity and stereospecificity. There a few examples, however, of CYP enzymes that exhibit some degree of non–stereo-specific hydroxylation, for example, the oxidation of norbornane by CYP2B1 (3).

Alabaflavenone monooxygenase was first identified from the soil bacterium *Streptomyces coelicolor* A3 (2), which is the most studied member of the *Streptomyces* genus of bacteria and produces a chemically diverse array of different secondary metabolites, including antibiotics, pigments, siderophores, hopanoids, and other lipids (4). CYP170A1 is a member of a two-gene operon also containing a sesquiterpene cyclase that converts farnesyl diphosphate (FPP) to the tricyclic hydrocarbon epi-isozizaene (5). This monooxygenase has been clearly shown to catalyze the conversion of the terpenoid epi-isozizaene to an epimeric mixture of albaflavenols, which are then oxidized to the sesquiterpene antibiotic albaflavenone (6). We observed nearly identical proportions of the two C5 hydroxylation epimers in the product albaflavenol, suggesting that there might be two distinct modes of binding the epi-isozizaene substrate. Although it is reasonable to assume that formation of the two epimeric albaflavenol intermediates arises from competing abstraction from each face of a saturated methylene and addition of an oxygen atom to form the chiral carbon center (3, 7), here we suggest a structural basis that emphasizes the essential role played by the precise geometry of substrate binding in determining the stereospecificity of cytochrome P450s. The crystal structure of CYP170A1 complexed with the substrate epi-isozizaene may allow us to understand further the chemistry and stereochemistry of substrate oxidation at the atomic level.

Another unique feature of CYP170A1 that is revealed in this study is the first example of a bifunctional cytochrome P450 containing a moonlighting terpene synthase active site in addition to a traditional monooxygenase active site. Bifunctional

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[S] The on-line version of this article (available at http://www.jbc.org) contains supplemental "Experimental Procedures," additional references, Tables S1 and S2, and Figs. S1–S3.

The atomic coordinates and structure factors (codes 3dbg and 3el3) have been deposited in the Protein Data Bank, Research Collaboratory for Structural Bioinformatics, Rutgers University, New Brunswick, NJ (http://www.rcsb.org/).

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[2] The abbreviations used are: CYP or P450, cytochrome P450; BisTris, 2-[bis(2-hydroxyethyl)amino]-2-(hydroxymethyl)propane-1,3-diol; FPP, farnesyl diphosphate; GC/MS, gas chromatography/mass spectrometry.

enzymes are found throughout the biological kingdoms, frequently involving sequential steps in metabolic pathways (8, 9). The presence of two active sites in a single polypeptide chain can accelerate the rate of conversion of the first product to the second product (10, 11). The most common type of bifunctional enzyme results from coupling of two polypeptide chains as a result of evolutionary fusing of two genes. In some cases, two such active sites whose functions appear to be unrelated have also been observed. Much less frequently observed are enzymes whose polypeptide sequence and length classify them as a specific protein class, yet contain a moonlighting active site from a different protein class inserted within their tertiary structure (12–15).

One of the most studied CYP enzymes, CYP102A1 (P450BM3) falls into the class of bifunctional enzymes by being a fusion between a cytochrome P450 and the well known eukaryotic-like microsomal P450 reductase (16). There are a few other examples of this type of bifunctional P450 in the same gene subfamily (CYP102A) and elsewhere in the superfamily. It is important to emphasize that P450s that metabolize widely different substrates via monooxygenase activities in a single heme-containing active site do not fit into the current definition of multifunctional enzymes. Rather, two distinct active sites are necessary for classification as a bifunctional enzyme.

CYP170A1 produces not only the antibiotic albaflavenone but also the acyclic sesquiterpene farnesene. Both enzymatic and structural data clearly demonstrate that CYP170A1 possesses two distinct active sites that catalyze two very different and probably unrelated biochemical activities. This study is the first to report the potential production of farnesenes by bacteria, possibly indicating new functions for these terpenes.

EXPERIMENTAL PROCEDURES

Crystallization, Data Collection, and Structure Determination—CYP170A1 was expressed and purified as reported earlier (6). Crystals of ligand-free CYP170A1 (native and selenomethionine-labeled proteins) and the epi-isozizaene complex were grown in hanging drops. All diffraction data were collected at the Southeast Regional Collaborative Access Team 21-ID beamline and the Life Science Collaborative Access Team beamline at the Advanced Photon Source, Argonne National Laboratory, Argonne, IL. The selenium sites were determined, and the electron density map of ligand-free CYP170A1 was obtained using single-wavelength anomalous diffraction phases. A 2.6-Å resolution model was obtained with the native protein. The epi-isozizaene complex structure was solved by molecular replacement at 3.3-Å resolution. The details and the final refinement statistics are given in supplemental "Experimental Procedures". The coordinates and associated structure factors have been deposited with the Protein Data Bank (identification codes 3DBG and 3EL3).

Sesquiterpene Synthase Assay in Vitro—For the terpene synthase activity, CYP170A1 (1 nmol) was assayed in 400 μl of 50 mM BisTris-HCl buffer (pH 5.5) containing 20% (v/v) glycerol and 10 nmol of FPP plus 5 mM Mg^{2+}. The reconstituted enzyme solution was placed in a shaking water bath at 30 °C. The reaction was carried out for 5 min in a 10-ml test-tube, at which time it was quenched and extracted three times with 400 μl of pentane-methylene chloride (4:1). The extracts were concentrated under a stream of N_2, and 2 μl of extract was analyzed by GC/MS (6). The kinetic parameters were determined by carrying out a series of 5-min incubations with substrate concentrations of 5–250 μM. The mixtures were extracted and analyzed by GC/MS. The K_m and k_{cat} were calculated by a nonlinear regression fit to the Michaelis-Menten equation using the GraphPad Prism software (GraphPad Software, San Diego, CA). The crude extracts from cells of *S. coelicolor* wild-type and CYP170A1 knock-out strains were prepared as described previously (6), but modified as: the cells were harvested by centrifugation and resuspended in 20 ml of lysis buffer (100 mM Tris-HCl buffer (pH 8.0), 10% (v/v) glycerol, and 5 mM $MgCl_2$) containing 20 mg of lysozyme and incubated at 25 °C for 1 h. Cells were broken by sonication, and the cytosol containing CYP170A1 was adjusted to pH 5.5 and then incubated in reaction buffer for 60 min.

Metal Cations for Farnesene Synthase Assay—To examine the different metal cofactor requirements for synthase activity, CYP170A1 was incubated in 400 μl of 50 mM BisTris-HCl buffer (pH 5.5) containing 20% (v/v) glycerol and 50 μM FPP. The different metal cations tested were Mg^{2+}, Mn^{2+}, Zn^{2+}, Fe^{2+}, Co^{2+}, Fe^{3+}, Ca^{2+}, Ni^{2+}, and Cu^{2+} at final concentration of 5 mM. In addition, the synthase activity was tested at different concentrations of Mn^{2+} (50 μM and 1 mM).

Effects of pH—The two different catalytic activities of CYP170A1 were investigated at different pH values from 4.5 to 9.0. The pH was adjusted at 25 °C. The enzyme was incubated for 15 min in buffers of different pH (50 mM). After incubation, a reduced CO difference spectrum was carried out using a double-beam Shimadzu UV-2401PC spectrophotometer to examine P450 stability (16). For synthase activity, the FPP was added at the end of the incubation period and the reaction carried out for 5 min. Catalytic activities were assayed as described above. For P450 monooxygenase activity, the reaction was carried out as reported previously (6).

Site-directed Mutagenesis—Two pairs of primers were used to make the DDXXD mutant D253A/D254A/D257A. Forward primer 1 was gctggaggcgaaggacGCCaatggcGCCccgatcggggaacagg, and reverse primer 1 was cctgttccccgatcggGGCgccattGGCgtccttcgcctccagc. Forward primer 2 was gctggaggcgaagGCCGCCaatggcGCCccgatcggggaacagg, and reverse primer 2 was cctgttccccgatcggGGCgccattGGCGGCcttcgcctccagc. Two steps were used to produce AA*XX*A. First primer pair 1 was used to generate the D254A/D257A mutant from wild-type CYP170A1 DNA. Then primer pair 2 was used to prepare the D253A/D254A/D257A mutant using the D254A/D257A mutant DNA as template. The PCR sample reaction is described in Ref. 1 as follows: 5 μl of 10× reaction buffer, 20 ng of template DNA, 125 ng of forward primer, 125 ng of reverse primer, 1 μl of dNTP mix, 4 μl of dimethyl sulfoxide, 34 μl of H_2O, and 1 μl (2.5 units) of *Pfu* turbo DNA polymerase. The reaction was carried out using cycling parameters: 95 °C for 30 s, 55 °C for 1 min, 68 °C for 7 min for 25 cycles, and 68 °C for 6 min. Then 40 μl of reaction to which 1 μl of DpnI restriction enzyme had been added was incubated at 37 °C for 2 h, after which 2 μl of the DpnI-treated reaction was transformed into 50 μl of DH5α cells. Following culture at 37 °C for 1 h, the cells were spread on

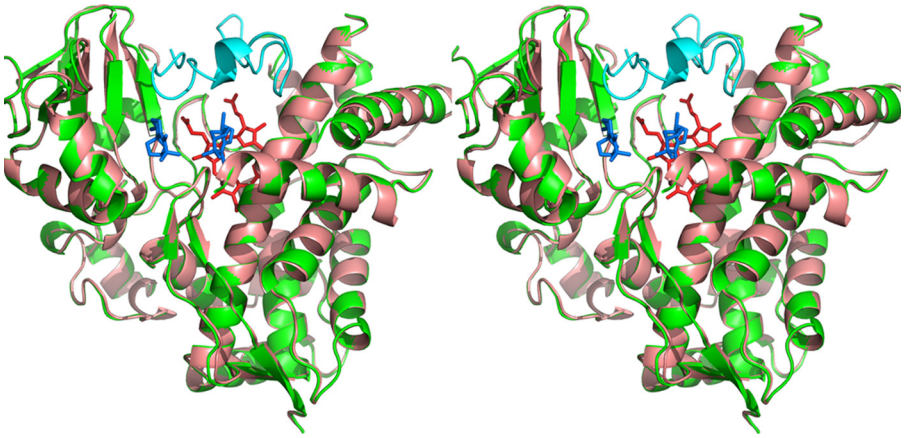

FIGURE 1. **Stereo view of overlay ribbon diagrams of CYP170A1 ligand-free and ligand-bound (epi-isozizaene) structures.** Both x-ray crystal structures show a typical cytochrome P450 fold. The ligand-free structure is represented in *salmon*, with the epi-isozizaene-bound structure in *marine*. Heme is shown as a *red stick model*. The BC loop in the structure of the epi-isozizaene complex is highlighted in *cyan*.

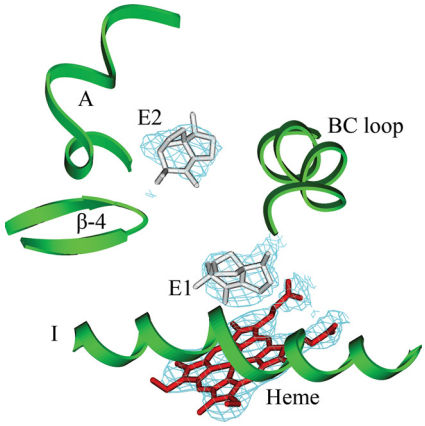

FIGURE 2. **Electron density for epi-isozizaene in the structure of substrate-bound CYP170A1.** The electron density map was calculated using σA-weighted $2|F_o| - |F_c|$ coefficients and is contoured at 1.0 σ. The electron densities above the heme are from two epi-isozizaene molecules. Heme is in *red*, epi-isozizaene in *gray*. Note that one epi-isozizaene molecule is situated in the proximal portion of the active site over the heme; the other is bound at the substrate entrance located between the BC and β-4 loops in a cleft on the distal surface of the protein. The distal epi-isozizaene is partially exposed to the bulk solvent and is ~8 Å away from the proximal substrate molecule.

an agar plate containing 100 μg/ml ampicillin. To test farnesene synthase activity, the mutant A253A254*XX*A257 was assayed as described above. To test albaflavenone monooxygenase activity, the reaction was carried out for 1.5 h in 400 μl of 50 mM Tris-HCl buffer (pH 8.2) containing 20% (v/v) glycerol and 20 nmol of epi-isozizaene plus 1% dimethyl sulfoxide as described previous (6). In kinetic studies, the assays were performed as reported earlier (6).

RESULTS

Structural Features of CYP170A1 Active Sites—To explore the structural basis for the albaflavenol epimers in the first step of hydroxylation of epi-isozizaene, x-ray crystal structures of CYP170A1, both ligand-free (pH 6.5) and epi-isozizaene-bound (pH 7.0) were determined at 2.6 and 3.3 Å, respectively. The overall structure of CYP170A1 exhibits the typical P450-fold consisting of α-helical and β-sheet domains as seen in all other known P450 structures (Fig. 1). At some loop regions in the ligand-free structure, a number of residues, 30 amino acids in the BC loop, 24 amino acids in the FG loop, and eight amino acids in the HI loop are disordered so that they could not be located in electron density maps. The BC and FG loops are associated with substrate recognition and binding in most P450 structures, and individually these regions are disordered in a few other P450s as well (17, 18). In the structure of the CYP170A1·epi-isozizaene complex (Fig. 1), the positions of amino acids in the BC loop are partially stabilized compared with these residues in the ligand-free structure. Some electron densities were observed in the BC loop region so that the model in the complex structure could be built. The residues in the BC loop dip into the active site to contact the substrate, but 16 amino acids in the FG loop and 8 amino acids in the HI loop are still disordered. Interestingly, this structure reveals that there are two bound molecules of epi-isozizaene that are clearly recognizable based on the electron densities (Fig. 2). One epi-isozizaene ligand is situated in the proximal portion of the monooxygenase active site over the heme; the other is bound at the substrate entrance located between the BC and β-4 loops in a cleft on the distal surface of the protein. This distal epi-isozizaene is partially exposed to the bulk solvent and is ~8 Å away from the proximal substrate molecule. It was not clear why two molecules of epi-isozizaene were bound to the single enzyme molecule. The substrate binding site is defined by several important residues that have been implicated in P450 substrate recognition and binding, with Trp-92, Pro-274, Val-338, Ile-447, and Thr-448 creating a hydrophobic cage over the heme. It was somewhat surprising that the two substrates were tilted at C5 so as to orient the exo or endo faces to the heme iron instead of binding the substrate in 180° opposite orientations. The existence of two major groups of substrate-binding modes in the two enzyme molecules might be consistent with the lack of stereospecificity in the oxidation of epi-isozizaene by CYP170A1, which results in the nonsterospecific hydroxylation leading to roughly equivalent amounts of the two epimers of albaflavenol (6). These structural features might favor the addition of oxygen atoms to both faces of the epi-isozizaene skeleton and production of both epimers of albaflavenol. The structure of CYP170A1 with bound epi-isozizaene agrees with the proposed mechanism of formation of hydroxy epimers in the oxidation of norbornane mediated by CYP2B1 (3). The subsequent oxidation of either albaflavenol epimer might involve intermediate release and rebinding steps leading to formation of the unstable gemdiol intermediate or relatively small motions of the initially formed albaflavenol intermediate in the active site leading to the net double-hydrogen abstraction mechanism.

CYP170A1 Sesquiterpene Synthase Activity—During initial studies of the monooxygenase activity of CYP170A1 an unex-

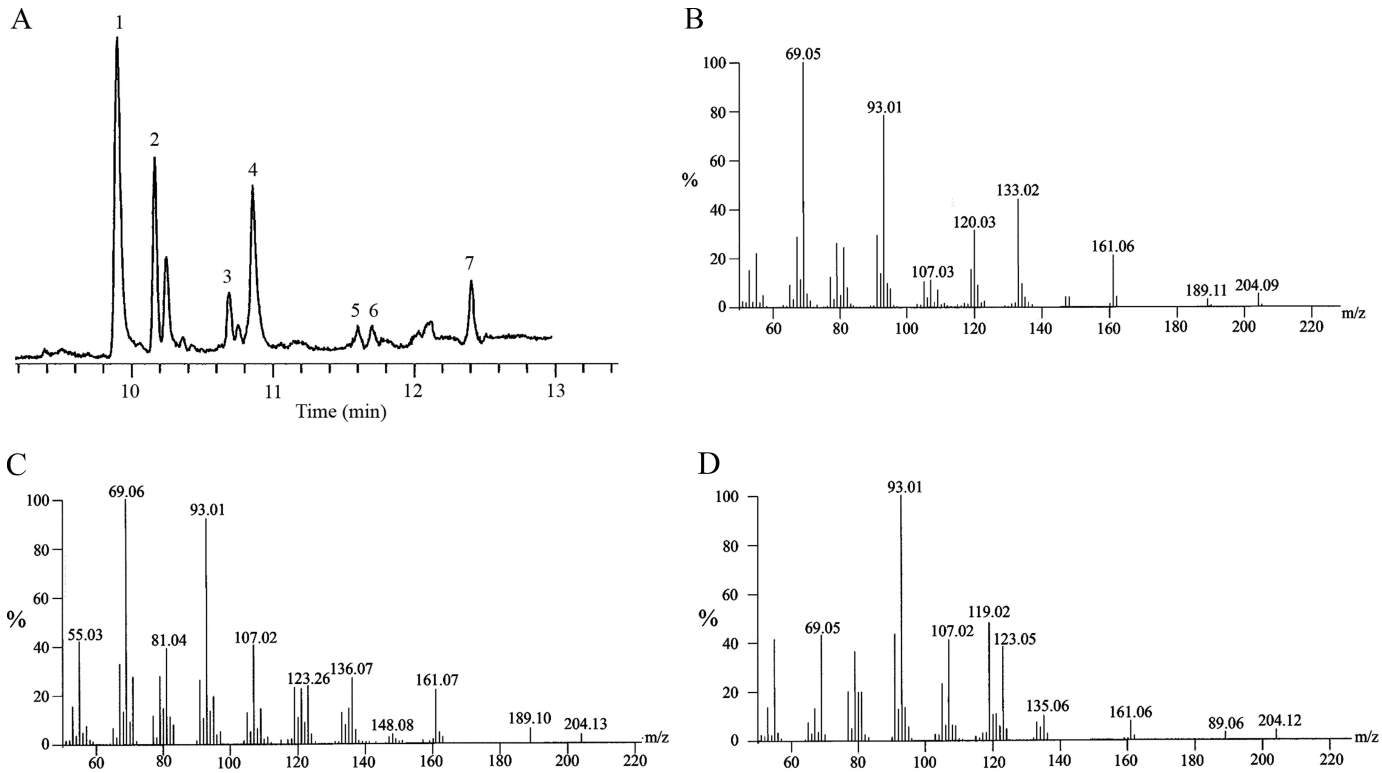

FIGURE 3. **GC/MS analysis of epi-isozizaene synthase and CYP170A1.** *A*, total ion chromatogram from GC/MS analysis of P450 and terpene synthase activity, as detailed under "Experimental Procedures." The products noted are: epi-isozizaene (*1*), (*E*)-β-farnesene (*2*), (3*E*,6*E*)-α-farnesene (*3*), (3*Z*,6*E*)-α-farnesene (*4*), the two epimers of albaflavenol (*5* and *6*), and albaflavenone (*7*). *B*, MS full scan of (*E*)-β-farnesene from incubation of FPP with CYP170A1. *C*, MS full scan of (3*Z*,6*E*)-α-farnesene from incubation of FPP with CYP170A1. *D*, MS full scan of (3*E*,6*E*)-α-farnesene from incubation of FPP with CYP170A1.

pected additional activity was also observed. Purified recombinant epi-isozizaene synthase and CYP170A1 were incubated with a CYP reductase system (*Escherichia coli* flavodoxin and flavodoxin reductase) and FPP, the substrate for epi-isozizaene synthase, to examine the expected CYP oxidation of the transiently generated epi-isozizaene. Surprisingly, GC/MS analysis of the products of the CYP170A1 incubation mixture revealed the formation not only of both epimers of albaflavenol and the fully oxidized sesquiterpene antibiotic albaflavenone (6), but also three unexpected sesquiterpene hydrocarbons that were evident in the total ion chromatogram with *m*/*z* 204, corresponding to $C_{15}H_{24}$ (Fig. 3). Formation of the *m*/*z* 204 terpenes was correlated with CYP170A1, as established by incubations with each of the individual components of the mixture as well as all combinations with 100 μM FPP (supplemental Table S1 and Fig. S1). Further, we have found that generation of these sesquiterpene products was independent of the presence of both redox protein partners (flavodoxin and flavodoxin reductase) and NADPH, suggesting that CYP170A1 had an intrinsic terpene synthase activity that was quite distinct from its monooxygenase activity required for production of albaflavenone.

By direct comparison of mass spectra of the individual products with spectra in the MassFinder 3.0 data base, the sesquiterpenes were identified as farnesene isomers (Fig. 4*A*) as confirmed by coinjection with authentic β-farnesene and both *trans*- and *cis*-α-farnesene standards. Remarkably, recombinant CYP170A1 alone displayed terpene synthase activity with FPP as substrate with GC/MS analysis of the products revealing formation of (*E*)-β-farnesene (61%), (3*E*,6*E*)-α-farnesene (26%), (3*Z*,6*E*)-α-farnesene (6.8%), as well as nerolidol (4.9%) and farnesol (1.8%) (Fig. 4*A*).

Cation and pH Requirements for CYP170A1 Terpene Synthase and Monooxygenase Activities—In some cases, sesquiterpene synthases can convert the lower and higher terpene substrate homolog, geranyl diphosphate or geranylgeranyl diphosphate, to monoterpene or diterpene products, respectively (19) in the presence of Mg^{2+} or Mn^{2+}. There were no detectable hydrocarbons formed, however, when either was tested as a surrogate substrate with CYP170A1 (supplemental Fig. S2*A*). The optimal kinetic parameters for CYP170A1-mediated sesquiterpene synthase activity were determined at pH 5.5. The k_m and k_{cat} values were calculated to be 16.8 μM and 0.019 s^{-1}, respectively, for FPP in the presence of Mg^{2+}, values that fall in the general range (0.01–0.3 s^{-1}) of other sesquiterpene synthases (20). The farnesene synthase activity of CYP170A1 has a strict requirement for a divalent cation cofactor (supplemental Table S1). The farnesene synthase activity was measured in the presence of a number of such cations (supplemental Fig. S2*B*), the highest activity being observed in the presence of Mg^{2+} or Ca^{2+}. Mn^{2+} shows relatively high activity at lower concentration, reaching 50% of the maximum synthase activity observed with Mg^{2+} as cofactor.

The effects of pH on both farnesene synthase and P450 monooxygenase activities were studied at different pH values between 4.5 and 9.0. The recombinant purified CYP170A1 exhibited maximum farnesene synthase activity between pH 5.5 and 6.5 (Fig. 4*B*). Plant and bacterial sesquiterpene synthases are generally fully active in the pH range of 6.5–7.5 (21).

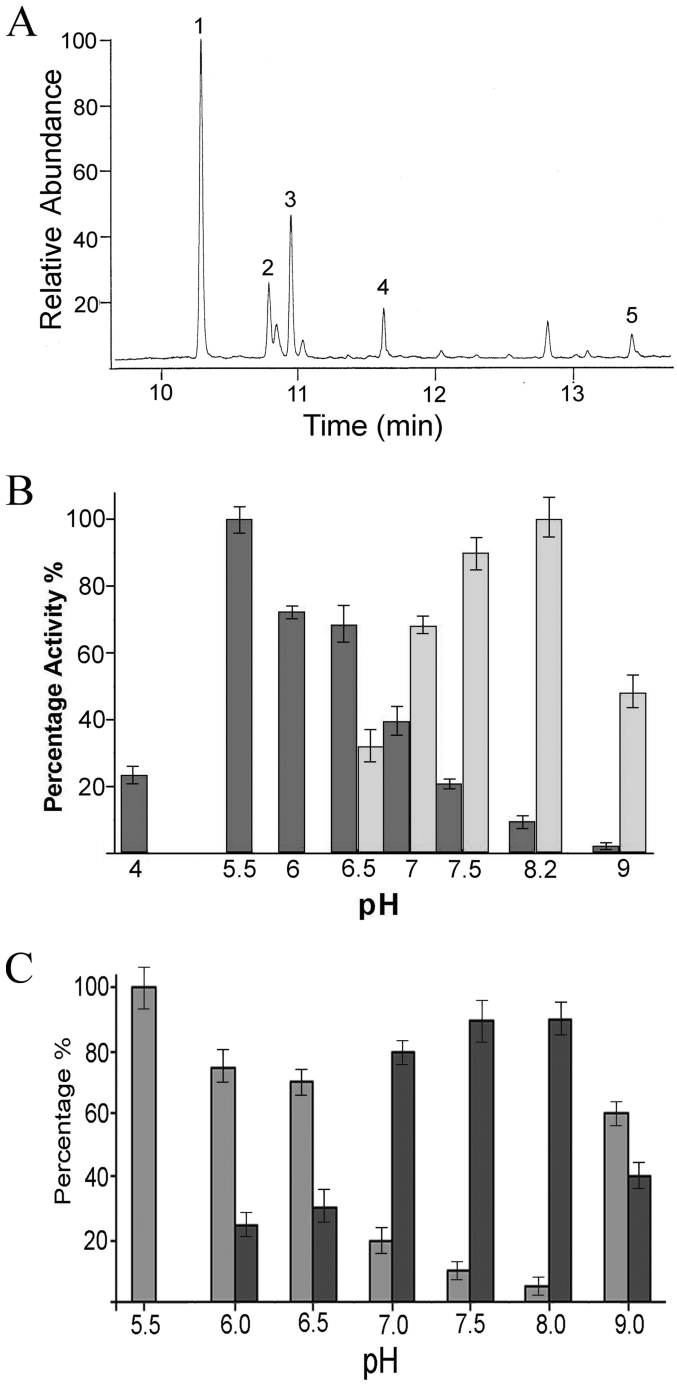

FIGURE 4. **Farnesene synthase activity of CYP170A1 and pH dependence.** *A*, total ion chromatogram from GC/MS analysis of products generated from FPP by CYP170A1. Three major products are noted: (*E*)-β-farnesene) (*1*), (3*E*,6*E*)-α-farnesene (*2*), (3*Z*,6*E*)-α-farnesene (*3*), and shunt products nerolidol (*4*), and farnesol (*5*) at pH 5.5. *B*, effect of pH on farnesene synthase activity (*dark gray bars*) and P450 monooxygenase activity (*light gray bars*). Reactions were carried out as described under "Experimental Procedures." *C*, effect of pH on level of P450 (active, *dark gray bars*) and P420 (inactive, *light gray bars*) forms of CYP170A1.

The monooxygenase activity of CYP170A1 is optimal between pH 7.0 and 8.2 (Fig. 4*B*). To assess the pH stability of CYP170A1, the enzyme was incubated at the indicated pH values in 50 mM buffer and the CO difference spectrum measured at 25 °C (22). Partially denatured forms of P450 were observed below pH 6.5 and above 9.0 (Fig. 4*C*). At pH 5.5, all of the P450 form of CYP170A1 was converted to the P420 form, which is consistent with the absence of residual monooxygenase activity at this pH. This decrease in monooxygenase activity at lower and/or higher pH is probably due to improper P450 folding. Thus, the monooxygenase activity declines at the lower pH, which favors sesquiterpene synthase activity, suggesting that the two different enzymatic activities possess their own optimal structural conformations.

The relationship between the sesquiterpene synthase activity and P450 monooxygenase activity at pH 7.0 was investigated by measuring the kinetic parameters for the two substrates, FPP and epi-isozizaene, *in vitro*. The results showed ~3-fold decreases in k_{cat} for both farnesene synthase activity and P450 monooxygenase activity when the two substrates were present at the same time. Whether both active sites are functioning in the same protein or if different populations of CYP170A1 catalyze only one of the reactions is not known. One possible explanation, however, is that binding either substrate (epi-isozizaene or FPP) to its cognate active site may drive conformational changes that would favor P450 monooxygenase activity or terpene synthase activity, respectively. Consequently, repositioning of amino acids induced by substrate binding could distort the other active site to some extent and thus cause the inhibition of its activity. To determine whether farnesene can channel between the two active sites of CYP170A1 and be further metabolized by the CYP170A1 monooxygenase activity, we titrated CYP170A1 with farnesene and found that the sesquiterpene hydrocarbon does not induce a typical type I P450 substrate binding spectrum, in contrast to the behavior of epi-isozizaene, the natural monooxygenase (6). In addition, there was no detectable formation of any new product when farnesene was added as a substrate.

β-Farnesene Synthase Activity in Cell Extracts—To explore whether the (*E*)-β-farnesene synthase activity of CYP170A1 is functional in cultured cells of *S. coelicolor*, the hydrophobic lipid extracts from both wild-type and a CYP170A1 knock-out strain were analyzed by GC/MS. Although (*E*)-β-farnesene was not directly detected in the crude culture extract under our experimental conditions, the relevant farnesene synthase activity could indeed be observed in the cytosol of wild-type *S. coelicolor*. Thus, incubation of crude cytosolic protein with added reaction buffer at pH 5.5 resulted in formation of (*E*)-β-farnesene, as detected by GC/MS (see "Experimental Procedures"), whereas terpene synthase activity was absent in the cytosolic extract of the CYP170A1 knock-out strain (supplemental Fig. S2*C*). These results suggest that there is sufficient endogenous FPP *in vivo* to allow farnesene formation catalyzed by endogenous CYP170A1. It is not clear, however, how and when the organism might trigger farnesene synthesis. Having two activities optimal at very different pH values, the organism could in principle regulate the enzyme function by sensing the environmental pH or could utilize the two CYP170A1 active sites at different stages of its life cycle.

Structural Basis of Sesquiterpene Synthase Activity of CYP170A1—To determine how CYP170A1 can possess the supernumerary sesquiterpene synthase activity, analysis of the primary sequence showed not only the standard P450 signatures (the highly conserved **E***XX***R** and **F***XX***G***XXX***C***X***G**(**S**)

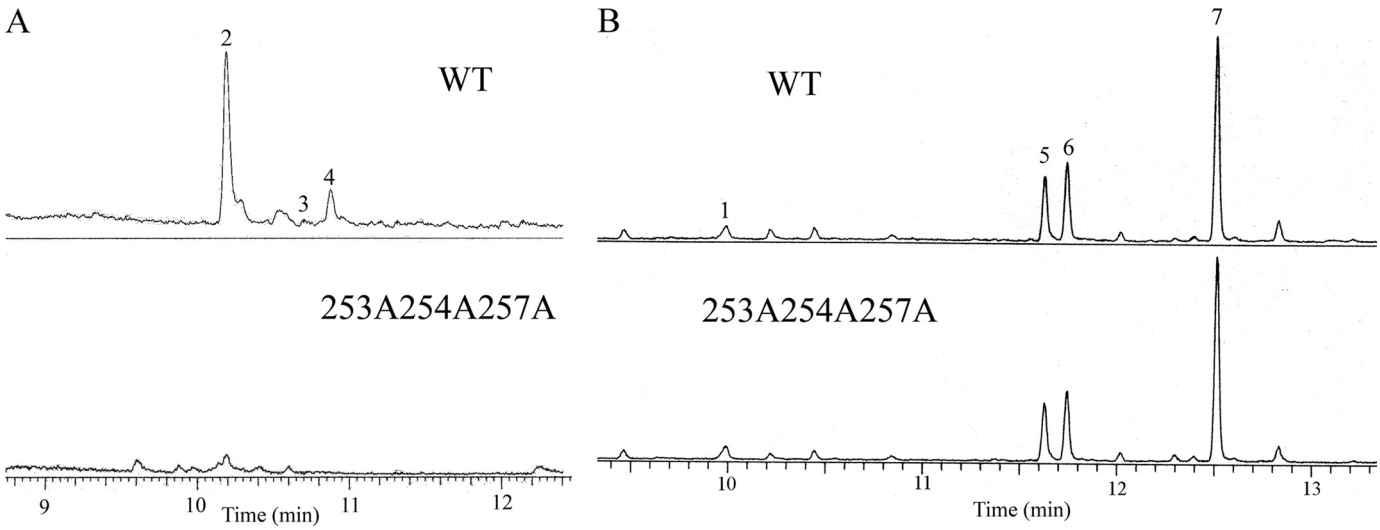

FIGURE 5. **GC/MS analysis of activities of CYP170A1 mutant D253A/D254A/D257A.** *A*, synthase activity of the D253A/D254A/D257A mutant. Three major products are noted: (*E*)-β-farnesene) (*2*), (3*E*,6*E*)-α-farnesene) (*3*), and (3*Z*,6*E*)-α-farnesene (*4*) in the wild type but not in the mutant. *B*, P450 monooxygenase activity of the mutant. The peaks noted are epi-isozizaene (*1*), two epimers of albaflavenol (*5* and *6*), and albaflavenone (*7*). The *upper panel* shows the product profile from wild-type (*WT*) enzyme, and the *bottom panel* is the product profile from the D253A/D254A/D257A triple mutant.

motifs), but also two putative Mg^{2+} binding motifs (the aspartate-rich sequence **DD***XX*(**D**/**E**) and the residues (**N**/**D**)D*XX*(**S**/**T**)*XXX***E**, (**NSE** or **DTE** triad) (supplemental Fig. S3), which are conserved in all terpene synthases of microbial and plant origin (23, 24). **DD***XX***D** is also found in numerous other proteins, including DNA and RNA polymerases (25). The other universally conserved terpene synthase signature sequence in CYP170A1 is **DTE**. The two conserved sequences are usually separated by ~140 amino acids, with **DD***XX***D** between amino acids 80 and 120 and **DTE** between positions 220 and 260 in microbial terpene synthases (26). In CYP170A1, however, **DD**NG**D** begins at Asp-253 and the **DTE** triad **DD**LL**T**ALL**E** at Asp-242. Site-directed mutagenesis of synthase active site residues converting all three (**DD**NG**D**) aspartate residues to alanines (D253A, D254A, and D257A) resulted in the loss of any detectable farnesene synthase activity (Fig. 5*A*), whereas the P450 monooxygenase activity was retained (Fig. 5*B*). The turnover number for epi-isozizaene oxidation in this mutant was estimated to be 0.31 ± 0.02 min^{-1} under the *in vitro* conditions used here, compared with (0.32 min^{-1} for the wild-type enzyme). Thus, the disordered **DD**NG**D** motif is essential for CYP170A1 farnesene synthase function, further confirming that there are two distinct active sites with different biochemical activities in the CYP170A protein.

Farnesene formation by CYP70A1 requires a divalent metal binding region in the terpene synthase active site. Several x-ray structures of sesquiterpene cyclases have been reported (27–30). They all share a similar overall terpenoid synthase structural fold of a hydrophobic barrel composed of six α-helices. In terpene synthase crystal structures, the two metal binding motifs together bind three divalent cations that in turn clamp in place and activate the pyrophosphate group of the FPP substrate. Attempts to generate complexes with Mg^{2+} and FPP or sodium diphosphate in the synthase active site of CYP170A1 failed because crystals cracked upon soaking, or no metal ions were observed bound to the conserved motifs in the structure. The CYP170A1 structures reveal a hydrophobic α-helical barrel domain including the C, H, I, and L helices (Fig. 6*A*). The aspartate-rich **DD**NG**D** motif is located on the HI loop at the entrance to the active site cleft, which contains eight disordered amino acids in both the ligand-free and epi-isozizaene-bound structures. The **DTE** triad motif is visible on the H helix. Several hydrophobic residues located inside the α-helical barrel might accommodate the geranyl tail of FPP (Fig. 6*B*). Arg-116 and Glu-263 coupled with the disordered **DDNGD** motif at the mouth of the barrel are believed to coordinate the divalent metal ion, which chelates to the diphosphate group of substrate. In the structure of epi-isozizaene-bound, the disordered HI loop containing a portion of the Mg^{2+} binding motif is consistent with the observed lower farnesene synthase activity when epi-isozizaene is present. Importantly, despite the general lack of overall amino acid sequence identity between CYP170A1 and other sesquiterpene synthases and the different location of the two conserved metal binding sequences in the primary structure, the three-dimensional structures of C, H, I, and L helices share a common α-helical barrel with other known synthases except that it contains four α-helices rather than six (Fig. 7). Also, most of the residues lining the active site cleft are very similar between CYP170A1 synthase and other sesquiterpene synthases (30). Notably, avian FPP synthase itself has no overall sequence-similar bimolecular electrophilic reactions of an allylic cation with a paired double bond (31).

DISCUSSION

The stereochemistry of cytochrome P450-catalyzed oxygenations has been extensively documented. Cytochrome P450cam (CYP101A1) catalyzes the single hydroxylation of camphor, producing only 5-exo-hydroxycamphor no matter whether native camphor or deuterated camphor derivatives is used as substrate (7), suggesting that hydrogen abstraction is not the key step in determination of product stereochemistry for CYP101A1 hydroxylation. Nonetheless, there is a significant isotope effect in the hydroxylation of norbornane by CYP2B1 (3). The structure of the CYP170A1·epi-isozizaene

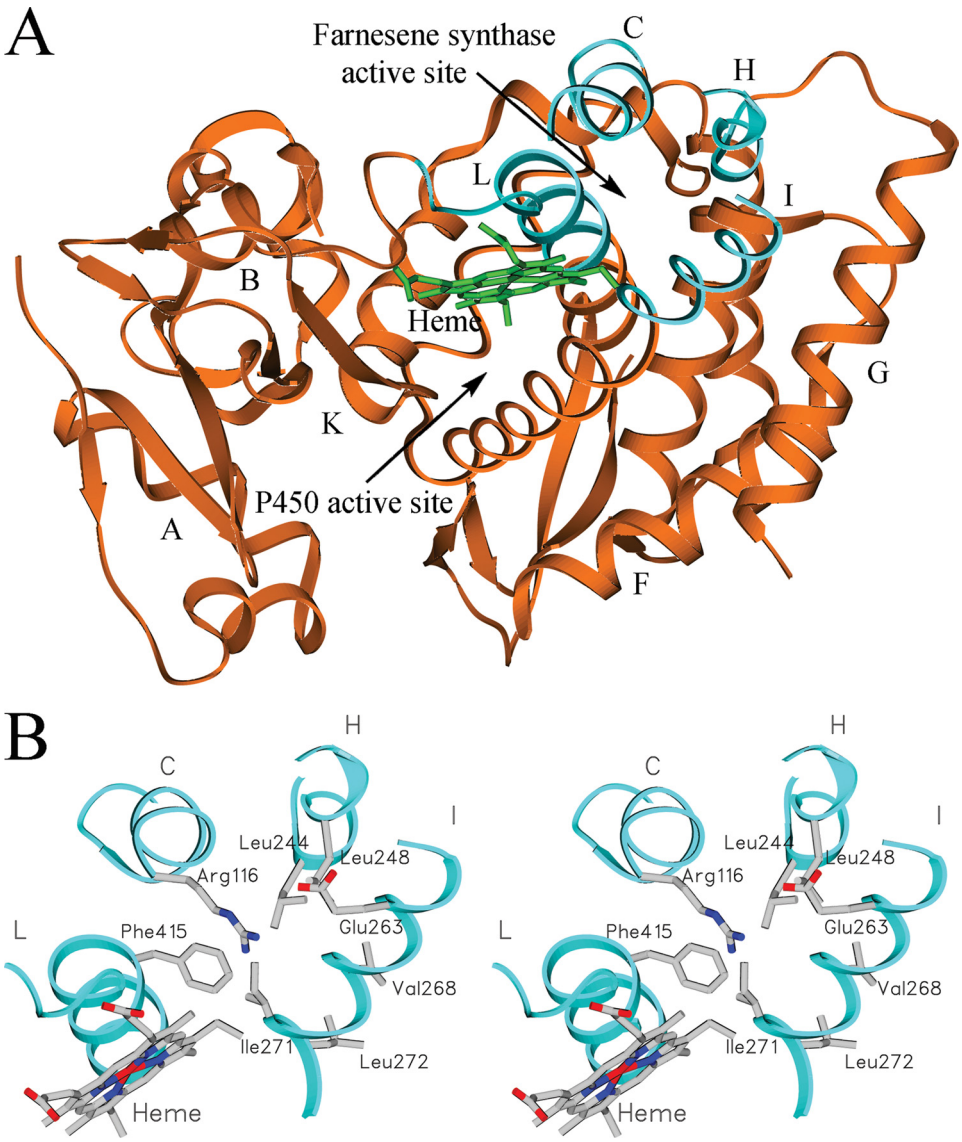

FIGURE 6. **Structures of farnesene synthase barrel in CYP170A1.** *A*, view of the farnesene synthase active site composed of C, H, I, and L helices. Heme is *green*. *B*, stereo view of farnesene synthase barrel region. Several hydrophobic residues (Leu2–44, Leu-248, Val-268, Leu-271, Ile-272, and Phe-415) are located inside the α-helical barrel, and Arg-116 and Glu-263 are at the mouth of the barrel. The side chain atoms and heme are rendered as *stick figures* (*gray*, carbon; *blue*, nitrogen; *red*, oxygen and iron).

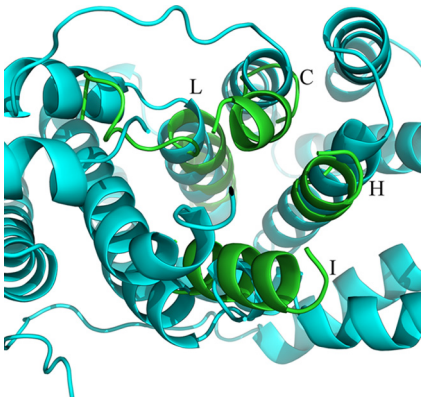

FIGURE 7. **Overlay ribbon diagrams of the synthase structural core of CYP170A1 and the aristolochene synthase.** The synthase structural core of CYP170A1 structure is represented in *green*, the helices from CYP170A1 are noted at *C*, *H*, *I*, and *L*, respectively; aristolochene synthase (41) is in *cyan*. Note that the global helical barrel in these two structures is very similar.

complex firmly suggests that geometrical positioning of the reactive carbon center or the radical intermediate in the enzyme active site is the major determinant of the stereospecificity of hydrogen atom abstraction and oxygen insertion in cytochrome P450. The complex of CYP101A1 with camphor exhibits only one substrate molecule occupied in the active site, with the exo face of C5 oriented toward the heme iron. Nevertheless, a question arising from comparing the activities of CYP170A1 and CYP101A1 is why CYP101A1 does not further oxidize 5-exo-hydroxycamphor to the corresponding 5-oxo-camphor? Presumably, the 5-endo hydrogen of 5-exo-hydroxycamphor is too far away from heme iron, and the active site cannot accommodate the binding of this product in an alternative orientation.

β-Farnesene produced by the moonlighting active site on CYP170A1 is an acyclic sesquiterpene olefin that has been found in both plants and insects and that can act as a defensive allomone (32), food attractant (33–35), and alarm pheromone (36, 37). Although there is extensive literature on the occurrence of this natural product and its deployment as an important courier in chemical communication in the plant kingdom, no known β-farnesene synthase genes have been found in bacteria, nor has β-farnesene ever been detected in bacterial extracts. There are many examples of bacterial enzymes that convert FPP to other terpenes (such as epi-isozizaene synthase), and FPP is synthesized in these bacteria (38). Bacteria can also metabolize farnesene, which in most cases is thought to be derived from plants (39). Although bacteria have not previously been reported to synthesize farnesene, there is no reason why they might not because a number of different secondary metabolites previously thought not to be produced in bacteria have recently been found to be produced in the bacterial kingdom (40).

CYP170A1, which displays both monooxygenase and farnesene synthase activity as disclosed here, is thus shown to be a very unusual enzyme. Most remarkable is that the farnesene synthase active site is harbored within a typical cytochrome P450 structure, containing well established heme-dependent monooxygenase activity. Thus, the sesquiterpene hydrocarbon epi-isozizaene is first converted to the individual epimers of albaflavenol, which are then in turn each converted to the same

ketone product, the antibiotic albaflavenone. On the surface of CYP170A1, ~20 Å away from the heme group is found the moonlighting terpene synthase active site located within the P450 α-helical domain found in all P450 structures at the top of the molecule based on the standard P450 view (Fig. 6*A*). Probably the reason that the two conserved cation binding sites are so close together in the primary sequence compared with all other terpene synthases is due to a four-helix active site. Furthermore, the HI loop forms a portion of the farnesene synthase active site. The disordered BC, HI, and FG loop regions conceivably are consistent with the observation of two different catalytic activities with distinct preferred pH ranges and may also indicate that the protein can switch between two different functional conformations. There are limited amino acids in the single polypeptide chain to fully form two distinct functional active sites so that the amino acids in the three mobile loops would assume different conformations associated with P450 monooxygenase and farnesene synthase activity. Nevertheless, as shown here, this is a functional terpene synthase active site. The structure of CYP170A1 as well as the differences in pH optima and the distance between the heme group and the metal binding site demonstrate that CYP170A1 is a rare bifunctional enzyme having two distinct active sites within a traditional P450 polypeptide chain that individually catalyze two apparently unrelated biochemical reactions. The presence of two such distinct biochemical activities in one protein is unprecedented within the P450 superfamily. Searches for the two conserved Mg^{2+} binding motifs among all known bacterial P450 primary sequences revealed that only the close ortholog CYP170A2 from *Streptomyces avermitilis*, which is also paired with an epi-isozizaene synthase, possesses both metal binding signatures in the farnesene synthase active site, suggesting that farnesene synthase activity could be specific to the CYP170A subfamily. (A few other bacterial CYPs contain one or the other of the conserved sequences but always outside the helical barrel.)

S. coelicolor CYP170A1 (and presumably *S. avermitilis* CYP170A2) is thus a unique enzyme in that it can produce two distinct secondary metabolites, one directly from FPP (farnesene) and the other indirectly from FPP (albaflavenone). A second novel feature of CYP170A1 is the profound difference in the pH optimum of the two associated biochemical activities. It is reasonable to propose that optimal activity of each of the two active sites depends on a different conformation of CYP170A1, the synthase activity being optimal when the monooxygenase active site assumes the denatured P420 form. It will be interesting to determine whether conversion of CYP170A1 to the P420 form by a method other than lowering the pH will optimize the terpene synthase activity. It is unknown whether different stages of the *S. coelicolor* life cycle have different internal or local pH values. Were this to be the case, it might be that farnesene and albaflavenone are not both produced at the same stage in the bacterial life cycle. Thus, CYP170A1 might have different active conformations in different stages of the parent organism.

Although such questions about CYP170A1 are interesting, the most curious is how did the moonlighting terpene synthase active site arise in CYP170A1 and why? The latter question cannot be addressed, but the former would seem to have an answer because CYP170A1 is structurally a very traditional cytochrome P450. As this P450 evolved from an ancestral monooxygenase by either divergent or horizontal DNA transfer and cellular protein engineering, the synthase active site could have arisen from a combination of P450 engineering (the positioning of the 4 helices) and additional engineering leading to an FPP binding site including binding sites for Mg^{2+}. Can this be just a freak of evolution, or is there a purpose for having the moonlighting synthase active site in this P450? We imagine this will be extremely difficult to answer and must await the discovery of other P450s having moonlighting active sites.

Given the many signaling and defense mechanisms associated with the farnesene molecule, it would be of great interest to find that a microorganism like a streptomycete potentially produces such a biologically important compound. The discovery of the heretofore cryptic farnesene synthase activity of CYP170A1 is a particularly striking example of the recent revelation by genomic analysis that soil microorganisms have the potential to produce a much greater range of secondary metabolites than have been uncovered by typical laboratory screening programs.

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