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Mutational analysis of a monoterpene synthase reaction: Altered catalysis through directed mutagenesis of (−)-pinene synthase from *Abies grandis* ☆

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Abstract

Two monoterpene synthases, (−)-pinene synthase and (−)-camphene synthase, from grand fir (*Abies grandis*) produce different product mixtures despite having highly homologous amino acid sequences and, presumably, very similar three-dimensional structures. The major product of (−)-camphene synthase, (−)-camphene, and the major products of (−)-pinene synthase, (−)-α-pinene, and (−)-β-pinene, arise through distinct mechanistic variations of the electrophilic reaction cascade that is common to terpenoid synthases. Structural modeling followed by directed mutagenesis in (−)-pinene synthase was used to replace selected amino acid residues with the corresponding residues from (−)-camphene synthase in an effort to identify the amino acids responsible for the catalytic differences. This approach produced an enzyme in which more than half of the product was channeled through an alternative pathway. It was also shown that several (−)-pinene synthase to (−)-camphene synthase amino acid substitutions were necessary before catalysis was significantly altered. The data support a model in which the collective action of many key amino acids, located both in and distant from the active site pocket, regulate the course of the electrophilic reaction cascade.

Keywords: Monoterpene synthases; Monoterpene cyclases; Terpene cyclase structure–function; Geranyl diphosphate cyclization; Turpentine biosynthesis; Directed mutagenesis; Pinene; Camphene; *Abies grandis*; Grand fir3

The abundant variety of terpenoids found in nature are all derived from common C_{10}, C_{15}, or C_{20} acyclic prenyl diphosphate precursors through the activity of the terpenoid synthases. Several thousand terpenoids are known, and much of this diversity arises from the activity of different enzymes employing variations on a common electrophilic reaction mechanism [1–3]. Individual enzymes also display catalytic variation, and many of the characterized terpenoid synthases produce multiple products. This proclivity of a single enzyme to produce multiple products is variable. Some enzymes, like 5-epi-aristolochene synthase from tobacco, produce essentially a single terpenoid product [4], while others, such as the γ-humulene and δ-selinene synthases from grand fir [5], produce more than 50 different products. Most terpenoid synthases fall somewhere in between these two extremes, producing a few predominant products and a number of minor products.

The first step in the terpenoid synthase reaction is the divalent cation-dependent ionization of the prenyl diphosphate precursor to create an enzyme bound allylic carbocation. This ionization step is common to all terpenoid synthases and, while there are some variations between synthase types, it generally proceeds via a common mechanism. The amino acid side chains

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thought to be involved in the ionization are usually conserved in all terpenoid synthases and include, for example, the DDxxD motif that is indicative of a terpenoid synthase. The latter part of the reaction is more variable and creates most of the product diversity. The allylic carbocation generated in the ionization step may undergo one or more cyclizations via the internal double bonds, methyl migrations, hydride shifts, or Wagner–Meerwein rearrangements before the reaction is terminated by deprotonation or nucleophile capture. In the case of the monoterpene (C_{10}) synthases, the precursor is geranyl diphosphate (GPP)[1] which is ionized and isomerized to enzyme bound linalyl diphosphate (LPP) to permit subsequent cyclization. Extensive study of the origin of enantiomeric *p*-menthane type monoterpenes (reviewed in [1] and [6]) has demonstrated a divergent, mirror-image pathway (Fig. 1) in which enzymes that produce the (+)-series of products convert GPP to the (3*R*)-linalyl intermediate, whereas enzymes that produce the (−)-series of products involve the (3*S*)-linalyl intermediate. After the LPP intermediate is formed, a second ionization and C1–C6 cyclization generates the corresponding α-terpinyl cation intermediate common to all cyclic monoterpenes. The reaction pathways for the different enzymes diverge after these initial steps to produce the various monoterpene products.

Although the above transformations have been described in considerable detail from the perspective of the geranyl precursor, there is much less information available on the role of the enzyme in the process. Recently, solved crystal structures of terpenoid synthases [7–12] have provided structural details of the active site pockets, the binding of various ligands, and the coordination arrangement of the diphosphate group with the requisite divalent cations. These studies have provided insight into the initial ionization step, and modeling of the reaction pathways within the experimentally determined active sites has led to plausible proposals for the course of selected reactions. However, many details of how the different synthases direct the reactions along specific channels leading to different products remain unclear. Domain swapping studies have addressed this question and have identified regions broadly responsible for product specificity [13–17]. Directed mutagenesis studies targeting specific residues have also been informative, in some cases leading to synthases with altered product outcomes [18–25]. In most of these cases, however, the mutations have led to a broadening of the product profile, or to the production of mechanistically simpler products, and have not identified specific mechanistic roles for individual amino acids.

Grand fir (*Abies grandis*), which possesses elaborate, terpenoid-based chemical defenses [27,28], has been a rich source of terpenoid synthases, and has provided examples of monoterpene [29,30], sesquiterpene [5,31], and diterpene [32] synthases. Of the monoterpene synthases, a group of seven enzymes has been characterized, each producing a different mixture of monoterpene products despite amino acid sequence identities ranging from 64 to 91% (pairwise comparisons). Because of the mechanistic differences among these closely related enzymes, they have been developed as a model system for understanding the mechanisms underlying product specificity. Two of these enzymes, (−)-pinene synthase (PS) and (−)-camphene synthase (CS), were chosen for the current study because they exhibit fundamental mechanistic differences despite sharing 77% amino acid sequence identity (Fig. 2). PS catalyzes Markovnikov addition to the double bond of the α-terpinyl cation (C2–C8 closure, Fig. 3) to produce the pinyl cation, from which alternative deprotonations give either (−)-α-pinene or (−)-β-pinene [29]. In contrast, the reaction pathway leading to camphene passes through the bornyl cation, which can arise either from rearrangement of the pinyl cation or through direct anti-Markovnikov addition (C1–C8 closure) to the double bond of the α-terpinyl cation.

In the present study, directed mutagenesis was used to replace amino acid residues in PS with the corresponding CS residues to define those responsible for mechanistic differences, and to better understand their role in catalysis. We report several mutants with variously altered activities, most notably mutated pinene synthases in which the majority of product passes through the alternative bornyl cation intermediate.

Materials and methods

Materials and substrates

The truncated clone for *Abies grandis* PS from which the plastidial targeting sequence was deleted [29] in the pSBET vector [33] was used as template for mutant construction. [1-^{3}H]GPP (100 Ci/mol) was prepared as previously described [34]. Unlabeled GPP was prepared from geranyl chloride as described in reference [35]. Monoterpene standards were from our own collection.

Structural modeling

Models of grand fir PS and CS [30] were generated by fitting the amino acid sequences to template structures of

[1] *Abbreviations used:* Bis-Tris, 1,3-bis[tris(hydroxymethyl)methylamino]propane; CS, (−)-camphene synthase; DTT, dithiothreitol; FID, flame ionization detection; GC, gas chromatography; GPP, geranyl diphosphate; Hepes, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid; IPTG, isopropyl-1-thio-β-D-galactopyranoside; LB, Luria–Bertani; LPP, linalyl diphosphate; MS, mass spectrometry; PAGE, polyacrylamide gel electrophoresis; PCR, polymerase chain reaction; PS, (−)-pinene synthase; SDS, sodium dodecyl sulfate.

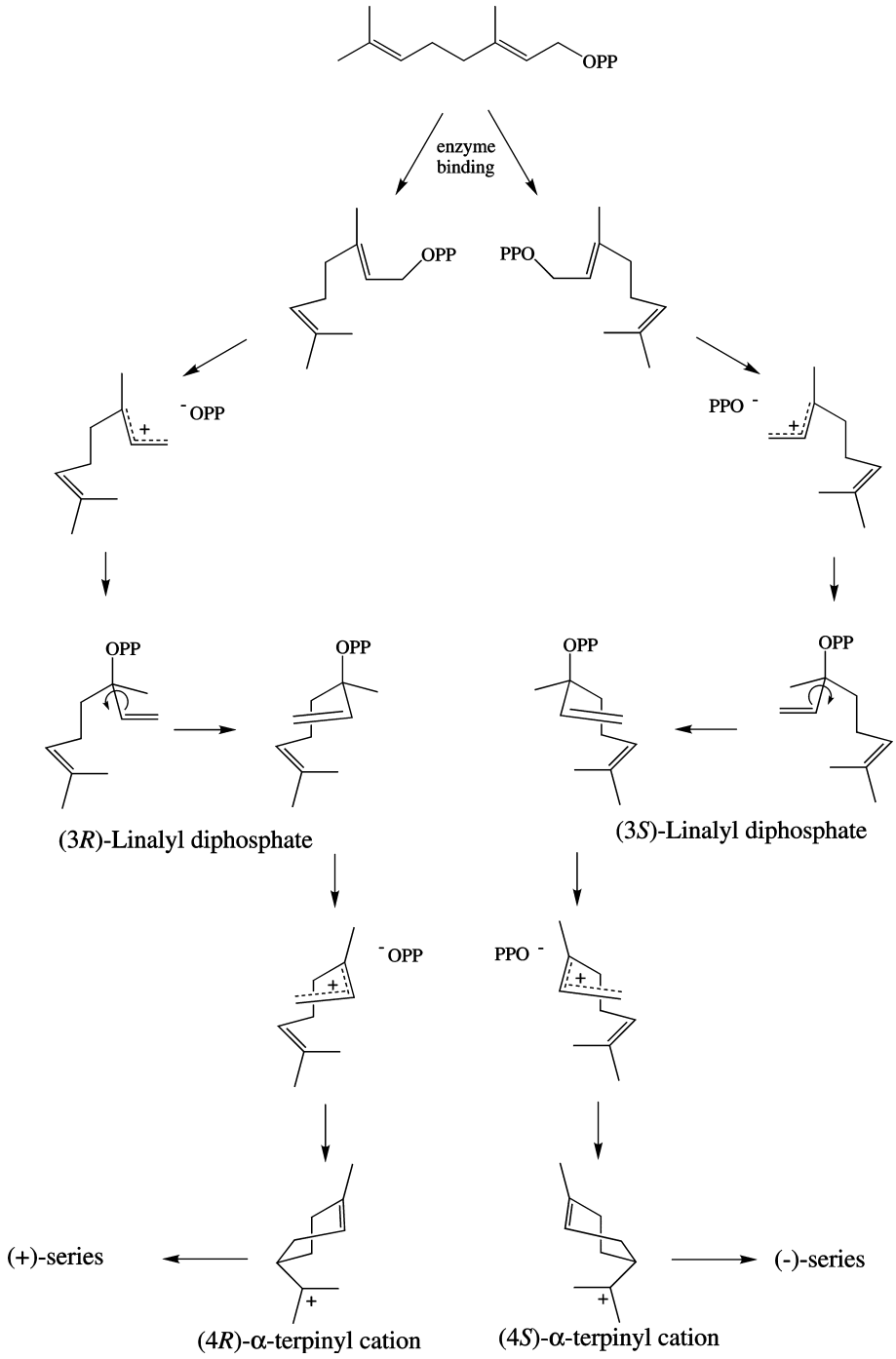

Fig. 1. Formation by monoterpene synthases of the central α-terpinyl cation intermediate of the (+)-series and (−)-series of monoterpenes (reviewed in [1] and [6]).

tobacco 5-epi-aristolochene synthase [8] or bornyl diphosphate synthase [12] through the Swiss-model server version 3.5 (http://www.expasy.ch), and the resulting models were visualized with the program O [36].

Mutagenesis and protein expression

Site specific mutants were made using PCR as previously described [17]. For generation of multiple point mutants, the procedure was carried out using the genes for previously mutated PS as template. All mutations were confirmed by gene sequencing. For expression, the pSBET vector carrying the mutated gene for PS was transformed into *Escherichia coli* BLR(DE3) cells, and the transformed cells were grown at 37 °C in 1 L of LB medium [37] supplemented with 50 μg kanamycin/mL. When the OD_{600} of the medium reached 0.5–0.8, the temperature was reduced and, after cooling (<25 °C), cul-

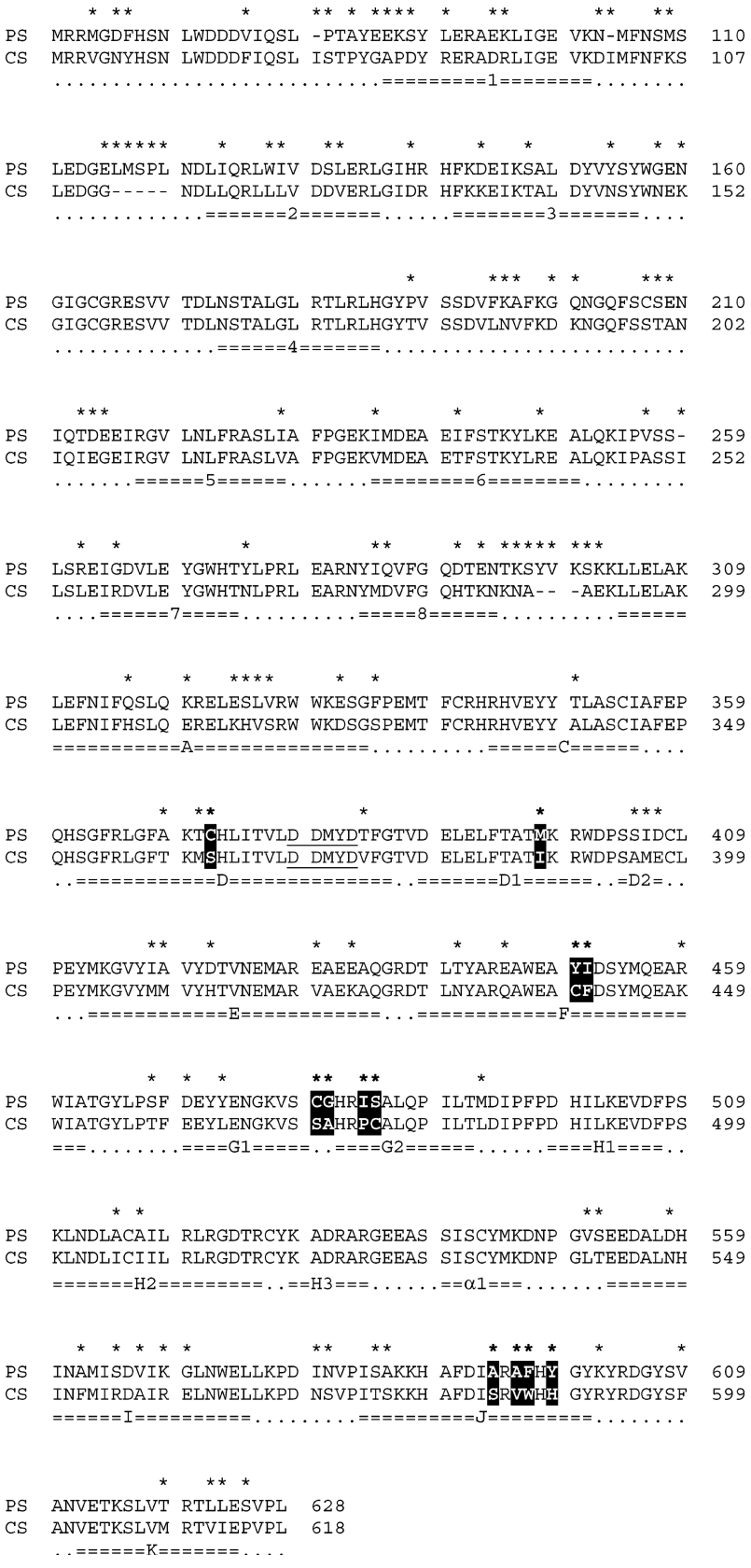

Fig. 2. Amino acid sequence alignment of truncated (plastidial transit peptide deleted) grand fir pinene synthase (PS) and camphene synthase (CS). Residues differing between the two enzymes are indicated with asterisks. Predicted secondary structure is indicated below the sequences with dots (…) for loops and double lines (= = =) for helices. The diphosphate and metal ion coordinating DDxxD motif is underlined. Residues mutated here and shown to contribute to altered catalysis are boxed in black.

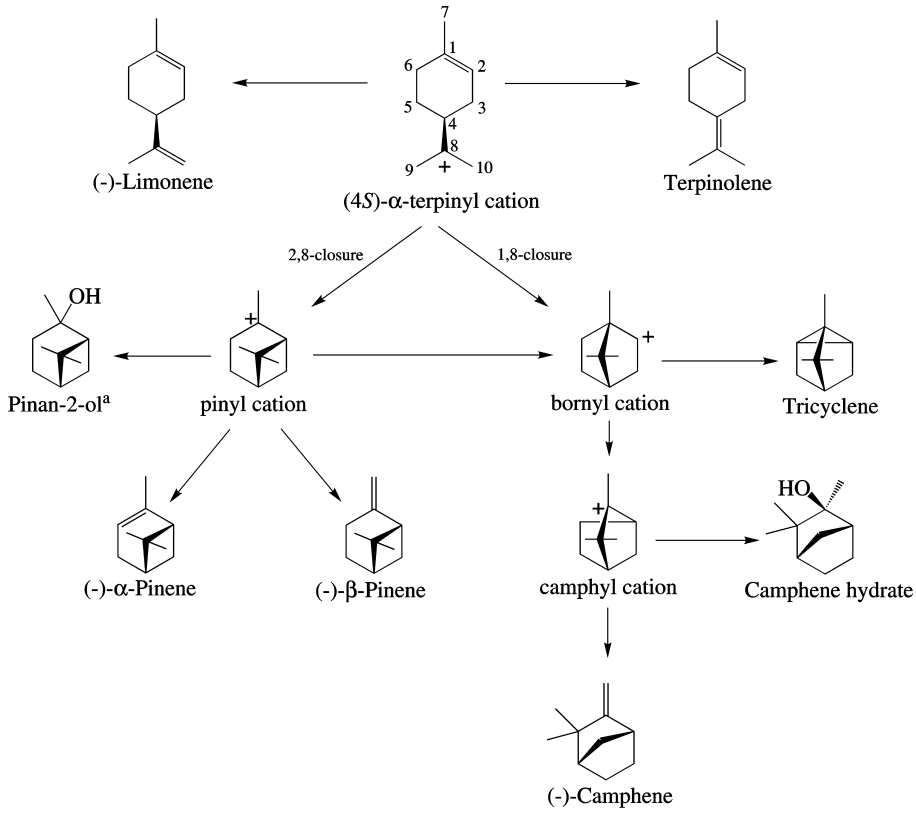

Fig. 3. Proposed pathways leading to the (−)-series monoterpene products of grand fir pinene synthase (PS) and its mutants. [a]Stereochemistry at C1 of pinan-2-ol is unknown.

tures were induced with 0.35 mM IPTG and grown for another 12 h at 20 °C. Cells were harvested by centrifugation (3500*g*, 15 min) and resuspended in 50 mL of cell wash buffer (50 mM Bis-Tris, 133 mM NaCl, pH 6.8). The resuspended cells were pelleted again (4500*g*, 15 min), the supernatant discarded, and the pellets were stored at −80 °C.

Enzyme isolation and purification

Frozen pellets of *E. coli* containing the overexpressed enzyme were thawed and resuspended in 50 mL of hydroxyapatite binding buffer (50 mM Bis-Tris, 5 mM $MgCl_2$, and 1 mM DTT, pH 6.8). The resuspended cells were lysed by sonication on an ice/ethanol bath (Virtis Virsonic, 0.5 in probe tip, max setting, 2 × 30 s). The cell lysate was clarified by centrifugation (11,000*g*, 20 min), and the enzyme was purified from the supernatant using a batch-mode hydroxyapatite binding step. In this procedure, 11.5 mL of supernatant was mixed in a 15 mL conical tube with 3.5 mL (dry volume) of ceramic hydroxyapatite (CHT type I, Bio-Rad) that had been equilibrated in binding buffer. The hydroxyapatite with bound enzyme was washed with three tube volumes of binding buffer, and the enzyme was desorbed with one tube volume of elution buffer (50 mM Bis-Tris, 10 mM sodium phosphate, and 1 mM DTT, pH 6.8). The resulting solution was desalted (Econo-Pac 10 DG, Bio-Rad) into 10 mM Hepes, pH 7.5. Aliquots were taken of the soluble and insoluble protein fractions, and of the hydroxyapatite-purified material, for SDS–PAGE analysis. This procedure typically yielded milligram quantities of enzyme at ~90% purity.

Enzyme assay and product analysis

A modification of a previously reported procedure [38] was used for determining comparative levels of substrate turnover in wildtype and mutant PS. In the present study, 10 μL of partially purified and desalted enzyme was mixed with 50 μL of 2× assay buffer (200 mM Hepes, 200 mM KCl, 2 mM $MnCl_2$, and 2 mM DTT, pH 7.5) and 38 μL H_2O in a 1.5 mL microfuge tube, and 2 μL of [1-^{3}H]GPP (450 μM, 100 Ci/mol) was added. The mixture was overlaid with 1 mL of pentane to trap volatile products. The mixture was kept on ice, and the reaction initiated by transfer to a 31 °C water bath with gentle agitation for 15 min, then vortexed and placed again on ice. The pentane overlay, and two additional 1 mL pentane extracts were combined and passed through a pasteur pipette containing silica gel (Mallinkrodt, Type 60 Å) and anhydrous $MgSO_4$ (2:1), and transferred into a scintillant-containing vial for counting. Two 1 mL volumes of diethyl ether were next

passed through the original silica gel/$MgSO_4$ column to elute oxygenated products, and this material was transferred to a fresh vial of scintillant. Radioactivity in the pentane and diethyl ether eluates was determined by counting in a Packard Tri-carb 2100TR liquid scintillation counter, and rates of conversion were determined from the specific activity of the substrate.

For product analysis, preparative amounts of both enzyme (~0.4 mg) and substrate (0.1 μmol) were used in a 2 mL assay to enable detection of minor products. Thus, 400 μL of purified and desalted enzyme was mixed with 1 mL of 2× assay buffer, 590 μL H_2O and 10 μL of ~10 mM unlabeled GPP in a glass screw-cap tube, and a 1 mL pentane overlay was added. The mixture was incubated in a 31 °C water bath with gentle agitation for 4–6 h, and then frozen. The reaction mixture was allowed to thaw without mixing, and the pentane overlay was transferred directly into a GC vial. Capillary GC-FID analysis was performed as previously described [17]. Products were quantitated by integration of the detector signal from 6 to 13 min which, under these parameters, spans the range of retention times of tricyclene (~6.3 min) through geraniol (~12.3 min). All other monoterpene products reported have retention times within this range. Products were tentatively identified by comparing retention times with those of authentic standards, and identities were confirmed by GC–MS analysis using previously described procedures [27]. Authentic standards for the minor products camphene hydrate and pinan-2-ol were not available; these products were identified by GC–MS comparison to library spectra (NIST Mass Spectral Library) and retention indices [39].

Results

In the present study, directed mutagenesis was used to replace selected amino acid residues in PS with the corresponding residues from CS to better understand catalysis by these two enzymes. Previous work has shown that PS produces 42% (−)-α-pinene and 58% (−)-β-pinene [29], and that the primary product of CS is camphene (54%) [30]. Because of the high sequence identity between these two enzymes, it was reasoned that reciprocal amino acid exchanges could define residues responsible for catalytic differences. The enzymes were overexpressed in *E. coli* and purified to ~90% homogeneity using hydroxyapatite, overall activity (substrate turnover/min) was determined using [1-^{3}H]GPP as substrate, and product analysis was done using unlabeled GPP followed by GC-FID and GC–MS. Large scale assay followed by product analysis of wildtype PS showed that, in addition to the previously observed mixture of α- and β-pinene (29 and 63% in this case), this enzyme also produces small amounts of myrcene (1.8%) and limonene (3.6%), along with trace amounts (<1%) of camphene, sabinene, terpinolene, linalool, and 4–6 unidentified products. A similar detailed analysis of wildtype CS, using large-scale preparations, was not possible because the bulk of CS was expressed as inclusion bodies in *E. coli* (soluble CS < 1% that of PS). For comparison, the five other monoterpene synthases from grand fir [29,30] were also similarly overexpressed in *E. coli*, and these enzymes displayed the same low level of soluble expression observed for CS. Of the group of seven enzymes, only PS was expressed as a soluble protein in sufficiently high levels for detailed product analysis. For this reason, all mutagenesis was done using PS as the parent enzyme.

Initial mutational analysis

PS and CS are highly homologous enzymes, most notably in the catalytic C-terminal domain[2] where the amino acid sequence identity is 82% (Fig. 2). Despite this homology, the differences (53 residues) are still numerous enough to preclude exhaustive mutagenesis at each position. To limit the number of residues for experimental consideration, the amino acid sequences of PS and CS were modeled onto the crystal structures of tobacco 5-epi-aristolochene synthase [8] and bornyl diphosphate synthase [12], and the resulting models were used as guides for selecting target residues. Residues that differed between the two enzymes and that were also predicted to reside in the active site pocket were the first targeted for mutagenesis.

Initial comparison of the PS and CS models identified four residues that appeared suitably positioned to influence catalysis. These residues were individually replaced in PS with the corresponding CS residues to make the C372S, C480S, S485C, and F597W point mutants. The overall activities (substrate turnover/min) of the mutants were comparable to wildtype PS. Product analysis showed that all of the mutants produced the same products as wildtype PS but with altered ratios of α- and β-pinene (Table 1). All four mutants produced an increased proportion of α-pinene and a correspondingly decreased proportion of β-pinene, while the levels of total pinenes (α- plus β-isomers) remained relatively constant (90–94%). The degree of this proportional shift varied with the different mutations and was most pronounced for the F597W mutant and least pronounced for the C372S mutant. The four mutants also produced increased proportions of minor products; notably, camphene and the mechanistically related camphene hydrate (Fig. 3). Also notable was the production of a trace amount (0.1%) of tricyclene by the C485S mutant.

[2] The catalytic domain is defined here as starting at residue 328, which corresponds to the approximate midpoint of domain-spanning helix-A.

Table 1
Initial point and multiple point mutations of pinene synthase (PS) and their product distributions and relative activities

Enzyme	Mutation				Percentage monoterpene product								
	C372S	C480S	S485C	F597W	Tricyclene	α-Pinene	Camphene	β-Pinene	Myrcene	Limonene	Camphene hydrate	Other	% Relative activity
PS						29.5	0.1	63.4	1.8	3.6		1.6	100
C372S	•					35.4	0.2	58.6	2.2	2.5	0.3	0.8	97
C480S		•				50.6	0.4	41.5	1.7	4.2	0.1	1.5	97
S485C			•		0.1	47.8	0.3	43.3	1.8	4.1	0.3	2.3	100
F597W				•		60.6	0.9	29.4	1.5	2.9	1.0	3.7	73
2-1			•	•	0.1	60.2	0.9	29.0	1.4	3.2	1.3	3.9	72
2-2	•		•		0.1	52.3	0.3	38.7	2.3	3.4	0.5	2.4	92
2-3	•	•				56.9	0.7	35.9	2.1	2.9	0.5	1.0	100
2-4		•	•		0.1	61.8	1.1	25.7	1.8	5.4	0.2	3.9	70
2-5		•		•		66.5	2.1	18.3	1.7	2.2	0.5	8.7	7[a]
2-6	•			•		62.9	1.0	24.9	2.3	3.1	1.4	4.4	68
3-1	•	•	•			69.2	1.2	19.4	1.8	4.3	1.0	3.1	77
3-2	•	•		•		73.7	2.4	10.7	1.6	4.2	1.1	6.3	74
3-3		•	•	•	0.2	73.1	2.6	12.1	1.1	4.3	1.7	4.9	73
3-4	•		•	•		61.9	0.9	24.7	2.3	2.6	2.6	5.0	100
4-1	•	•	•	•	0.1	79.5	2.4	10.4	1.7	3.1	0.9	1.9	99

[a] Low activity of mutant #2-5 is largely due to poor expression. Dots indicate substituted position. Activity levels are relative to wildtype PS. No entry indicates product not detected.

These increases in mechanistically related minor products (of the camphyl type), although small, were consistently observed with certain mutations throughout this study, and, in many cases, the increases were cumulative as certain mutations were combined (discussed below).

To determine the effects of multiple combined mutations, the C372S, C480S, S485C, and F597W mutations were combined into all six possible double mutant combinations, all four possible triple mutant combinations, and the quadruple mutant involving all four substitutions (Table 1). In general, the effect of combining mutations was additive, and all enzymes followed the same general trend in which the proportion of α-pinene increased at the apparent expense of β-pinene, and the proportion of camphene and camphene hydrate increased. This trend consistently grew more pronounced as additional point mutations were included but varied with different mutation combinations. The most subtle alteration of these was the S485C/F597W (#2-1)[3] combination, in which the proportions of camphene, α-pinene, and β-pinene were unchanged from the F597W single mutant. The only additive effect observed for mutant #2-1 was a small increase in the proportion of camphene hydrate. The additive effect observed for the C372S/S485C double mutant (#2-2) was also subtle. In this case, the proportion of camphene produced was unchanged from the S485C single mutant but the proportion of camphene hydrate produced was slightly higher (0.5%), and there was a slightly larger shift in the α- to β-pinene ratio than in either single mutant. The other four double mutants, C372S/C480S (#2-3), C480S/S485C (#2-4), C480S/F597W (#2-5), and C372S/F597W (#2-6), all displayed additive shifts in the α- to β-pinene ratios and additive increases in the proportions of camphene.

The additive effect of mutation continued to be observed when a third mutation was included to make the four triple mutant combinations. Three of the triple mutants, C372S/C480S/S485C (#3-1), C372S/C480S/F597W (#3-2), and C480S/S485C/F597W (#3-3), produced increased proportions of camphene and camphene hydrate as well as increased shifts in the α- to β-pinene ratios when the third mutation was included. For the C372S/S485C/F597W triple mutant(#3-4), product outcome was only slightly changed from that of the S485C/F597W (#2-1) double mutant, except for a doubling in the proportion of camphene hydrate (to 2.6%). When all four mutations were combined to produce the quadruple mutant, C372S/C480S/S485C/F597W (#4-1), there was not an observed increase in camphene or camphene hydrate and this mutant produced slightly less of these products than either #3-2 or #3-3; however, the α- to β-pinene ratio did continue to shift as the fourth mutation was added, resulting in a ratio of 80% α-pinene to 10% β-pinene.

The results of this initial analysis indicated that these four residues in the predicted active site of PS do indeed influence the reaction channel. Single point mutations had only a modest effect, but the effect became more pronounced as multiple mutations were combined. Of this initial set, the C480S and F597W mutations had the strongest influence on product out-

[3] In the numbering system for multiple point mutants, the first number is the number of mutated residues and the second number is a unique identifier. For example, #2-1 represents the first enzyme with two mutations.

come without appreciable influence on rate. When either of these mutations was made (to wildtype PS or to an existing mutant), the proportion of camphene increased by an average of 1.1%. In contrast, the other two point mutations, C372S and S485C, increased the proportion of camphene by 0.1 and 0.3%, respectively. In some cases, the effect of combining mutations was synergistic, particularly when the C480S or F597W mutations were involved.

Segment exchange mutants

The initial mutational analysis identified four residues that influence product outcome in PS. Two of these residues, Cys480 and Ser485, are located in a short peptide segment near the junction of the predicted G1 and G2 helices (helix labels are described in [8]). This region lines the predicted active site pocket and is variable among otherwise closely related monoterpene synthases, making it an attractive target for further mutagenesis. There are four amino acid differences between PS and CS within this six residue segment, so exchange of the region in PS with the corresponding CS region resulted in a quadruple mutant, C480S/G481A/I484P/S485C (#4-2). As with the previously described mutants, mutant #4-2 produced an increased proportion of α-pinene (to 78%), a decreased proportion of β-pinene (to 12%), and an increased proportion of minor products, notably camphene (to 1.6%) (Table 2). The change in product outcome was greater for this mutant, in which the entire 480–485 segment was exchanged, than for the C480S/S485C double mutant (#2-4), indicating that the additional G481A and I484P mutations also influence the reaction.

A second region targeted for exchange was a seven amino acid segment from position 594 to 599 that includes Phe597, which had been previously shown to influence catalysis. There are four amino acid differences between PS and CS within this region, so the exchange resulted in the A594S/A596V/F597W/Y599H mutant (#4-3). As observed with the previous segment exchange mutant, exchange of the entire segment influenced product outcome more than the F597W single mutation, even though the surrounding residues are not predicted to project into the active site pocket.

Further structural modeling of PS and CS revealed a third region of potential interest, located near the end of the predicted F-helix and adjacent to the active site pocket. This two-residue segment, position 450 and 451, appeared to vary enough between the two enzymes to influence the shape of the pocket and thus indirectly influence product outcome. Exchange of the 450 and 451 residues from CS into PS produced the Y450C/I451F double mutant (#2-7). Again, product outcome was altered, but in a manner different from previous mutations. In this case, the α- to β-pinene ratio was shifted toward β-pinene, and the proportion of α-pinene was reduced to 17%. There was also a significant increase in the proportion of limonene (to 8.5%) that was not observed with previous mutations, and there were increases in the proportions of several minor products.

Combining all mutations

To determine if the additive and synergistic effects observed with single point mutations could be extended to the segment exchange mutations, a strategy was adopted whereby all of the previously identified mutations (point mutations and segment exchanges) were combined in multiple combinations; point mutations were made to the three segment exchange mutants, segment exchange mutations were combined, and, finally, all influential mutations were combined together in a single enzyme. The results of this approach (Table 2) were similar to those when single point mutations were combined but more pronounced. In most mutation combinations, there was an increase in the proportion of α-pinene with a corresponding decrease in β-pinene but this effect plateaued once the proportion of β-pinene dropped below 5–10%, and the predominant effect of further mutation was an increase in the proportion of bornyl cation-derived products. The extent of these effects varied with the different combinations but, in general, combining mutations produced additive and often synergistic results.

Another generally observed result of combining mutations was that exchange of a short segment influenced product outcome more than the exchange of a single amino acid within the segment. An example of this is the result with mutants #7-2 and #10-1. Mutant #7-2 was made by making the F597W mutation to the mutant #6-3 parent enzyme. This single residue exchange in an enzyme already possessing six mutations significantly altered product outcome by increasing the proportion of bornyl cation-derived products from 12.4% (in mutant #6-3) to 33.5% (in mutant #7-2). However, exchange of the entire residue 594-599 region was more effective in altering product outcome, resulting in an enzyme (mutant #10-1) that produces 39.0% bornyl cation-derived products.

The synergistic effect of combining mutations generally became more pronounced as the total number of mutations increased. For example, the C372S mutation, when made to wildtype PS, influenced product outcome only slightly but when this mutation was made to an enzyme already altered by mutagenesis, the influence was considerably larger. Thus, when the C372S mutation was made to mutant #8-1 (producing mutant #9-1), the proportion of bornyl cation-derived products increased from 8.4 to 11.5%, and when made to mutant #10-1

Table 2
Multiple combined mutants of pinene synthase (PS) and their product distributions and relative activities

Enzyme	Mutation												Percentage monoterpene product								
	C372S	M398I	Y450C	I451F	C480S	G481A	I484P	S485C	A594S	A596V	F597W	Y599H	Tricyclene	α-Pinene	Camphene	β-Pinene	Myrcene	Limonene	Camphene hydrate	Other	% Relative activity
PS														29.5	0.1	63.4	1.8	3.6		1.6	100
2-7			•	•										16.9	0.4	65.5	1.6	8.5	0.7	6.4	88
3-5	•		•	•									0.1	17.2	0.3	68.0	1.6	5.6	1.4	5.8	92
3-6			•	•				•					0.2	41.5	0.9	35.9	1.1	7.2	2.5	10.7	100
3-7			•	•	•								0.1	32.8	1.0	45.0	1.5	9.7	1.3	8.6	52
3-8			•	•							•		0.1	30.9	0.9	40.7	1.6	6.0	4.2	15.6	10[a]
4-2					•	•	•	•						78.1	1.6	11.6	1.6	3.2	0.5	3.4	68
4-3									•	•	•	•	0.1	61.4	1.2	30.1	1.4	2.9	1.0	1.3	57
4-4			•	•	•						•		0.3	48.3	4.5	21.2	1.5	7.7	4.2	12.3	71
4-5	•		•	•							•			33.2	1.2	35.0	1.8	4.8	5.2	18.8	55
4-6			•	•	•			•					0.4	56.4	2.8	14.1	0.8	7.7	4.7	13.1	86
5-1					•				•	•	•	•		73.7	3.0	13.7	1.3	4.1	1.2	3.0	83
5-2					•	•	•	•			•		0.4	83.1	3.4	4.4	0.9	1.6	0.7	5.5	11
5-3	•				•	•	•	•					0.2	72.8	2.3	9.8	2.6	2.2	1.4	8.7	60
5-4								•	•	•	•	•	0.1	55.9	1.3	28.7	1.5	3.5	2.2	6.8	59
6-1	•				•	•	•	•			•		0.3	83.6	4.4	4.7	1.7	1.5	1.0	2.8	14
6-2			•	•					•	•	•	•	0.2	39.5	1.2	32.2	1.7	6.0	4.7	14.5	46
6-3			•	•	•	•	•	•					0.8	69.5	7.0	6.1	0.9	5.9	4.6	5.2	91
7-1	•		•	•	•	•	•	•					0.7	57.3	9.3	4.4	1.0	4.8	14.0	8.5	77
7-2			•	•	•	•	•	•			•		1.7	51.6	22.2	3.4	0.7	4.2	9.6	6.6	27[b]
7-3			•	•	•				•	•	•	•	0.4	52.3	5.5	14.4	1.3	7.6	5.9	12.6	53
8-1					•	•	•	•	•	•	•	•	0.7	78.5	6.7	6.5	1.2	1.8	1.0	3.6	3[b]
8-2	•		•	•	•	•	•	•			•		1.8	40.6	26.2	2.2	0.9	3.3	20.8	4.2	65
9-1	•				•	•	•	•	•	•	•	•	0.6	74.1	8.3	4.5	2.0	1.8	2.6	6.1	10
10-1			•	•	•	•	•	•	•	•	•	•	1.8	43.8	23.9	2.6	0.8	4.5	13.3	9.3	41
11-1	•		•	•	•	•	•	•	•	•	•	•	2.2	32.0	30.1	1.7	1.0	3.7	21.3	8.0	36
12-1	•	•	•	•	•	•	•	•	•	•	•	•	2.1	30.7	30.1	1.9	1.0	3.6	22.3	8.3	38

[a] Enzyme largely insoluble.

[b] About half of the expressed protein is insoluble. Dots indicate substituted position. Activity levels are relative to wildtype PS. No entry indicates product not detected.

(producing mutant #11-1), the proportion of bornyl cation-derived products increased from 39.0 to 53.6%. Mutant #11-1 is also significant in that it includes all of the mutations described above, and it channels more than half of the product through the alternative bornyl cation route.

Continued mutagenesis

Combining all of the previously described mutations in a single enzyme (mutant #11-1) resulted in a product profile that was significantly altered; however, the product profile of mutant #11-1 did not exactly match that reported for CS [30], suggesting that further mutagenesis might continue to alter catalysis. Structural modeling identified a number of additional PS to CS mutations that might further influence the reaction, and some of these were made to the mutant #11-1 parent enzyme. The M398I mutation was chosen because Met398 is predicted to be within van der Waals contact of Tyr450 and Ile451, both of which had proven to be influential. When the M398I mutation was made to mutant #11-1 (producing #12-1), there was a slight increase in the proportions of bornyl cation-derived products (from 53.6 to 54.5%).

A number of other PS to CS mutations were made that did not produce a more CS-like product outcome (data not shown). A segment exchange from position 369 to 372, which includes the influential C372S mutation, resulted in reduced soluble expression, as did a residue 515–517 segment exchange. An additional point mutation, M493L, did not affect enzyme solubility but led to slightly lower proportions of bornyl cation-derived products. A V609F point mutation led to both reduced soluble expression and a markedly reduced proportion of bornyl cation-derived products. These results indicate that not all PS to CS mutations result in a more CS-like product outcome.

The inability to locate additional residues that could significantly alter the reaction suggested that most of the residues responsible for the catalytic differences between PS and CS had been identified, although the two most altered enzymes (#11-1 and #12-1) did not generate a product mix matching CS exactly. It was assumed that some combination of the remaining amino acid differences in the C-terminal domain (35 residues) would incrementally alter product outcome to match that of CS. To test this idea, the entire C-terminal domain (from position 328 to the C-terminus) of PS was replaced with the CS C-terminal domain (analogous to making 53 point mutations to PS). The resulting chimeric enzyme was essentially insoluble, but the soluble form was active and produced large amounts of linalool (22.9%) and geraniol (36.8%). Interestingly, if just the olefinic products of this enzyme are considered, α-pinene is still most abundant and product outcome is very similar to that of PS mutants #11-1 and #12-1, rather than matching the product profile of CS.

Discussion

The initial results of PS to CS amino acid replacements were unexpectedly subtle and involved changes in the relative proportions of α- and β-pinene along with very slight increases in the proportions of bornyl cation-derived products. Although these results do not conclusively identify residues with specific roles, the pattern of changes in the α- to β-pinene ratios are consistent with a catalytic model in which Ser485 and Cys480 of PS act as terminal proton acceptors in the production of α- and β-pinene, respectively. Structural modeling shows that the pinyl cation intermediate can be positioned in a geometry suited for the relevant proton abstractions (Fig. 4). In this arrangement, a C7 proton from the pinyl intermediate (using numbering from the α-terpinyl cation) is aligned for abstraction by Sγ of Cys480 and a C6 proton is aligned for abstraction by Oγ of Ser485. Sulfur is a more efficient proton acceptor, either because of its greater nucleophilicity or because it is more readily ionized to a thiolate, and this could explain why wildtype PS produces more β-pinene than α-pinene. In the case of the C480S mutation, the sulfur of residue 480 is replaced with a less nucleophilic oxygen atom, resulting in reduced C7 deprotonation and a correspondingly reduced proportion of β-pinene. The opposite effect occurs with the S485C mutation, in which Oγ of residue 485 is replaced with a more nucleophilic sulfur, resulting in increased C6 deprotonation and an increased proportion of α-pinene in the product mixture. The combined effects would be expected in the C480S/S485C double mutant (#2-4), and indeed this enzyme produces an α- to

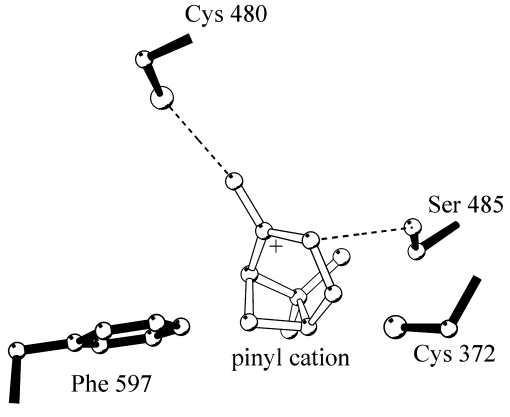

Fig. 4. Portion of the active site pocket of the wildtype pinene synthase model with the pinyl cation intermediate modeled into position for interaction with Cys480 and Ser485. The interaction between the amino acid side chain and the abstracted protons is indicated with a dashed line. This figure and Fig. 5 were generated with the program *Molscript* [42].

β-pinene ratio approximately reversed from that of wildtype PS.

While the data are consistent with the above deprotonation scheme, there are other groups that could potentially serve as transient proton acceptors, such as main chain carbonyl oxygens, ordered water molecules [12], or other amino acid side chains. The reaction likely follows a probabilistic route in which the active site environment favors abstraction by a specific group, but does not preclude abstraction by alternative groups. When Ser485 is replaced with alanine (unpublished result), substrate turnover is significantly reduced (to ~2% of wildtype), but the mutant enzyme can still produce both α- and β-pinene in an extended assay.

The results of the C372S and F597W mutations are more difficult to interpret but the locations of these residues in the PS structural model suggest that they could influence the interaction of the pinyl cation intermediate with the side chains of residues 480 and 485. Positional modeling of the pinyl cation for the deprotonations described above places this intermediate between residues 372 and 597 (Fig. 4). The side chains of these two residues may influence the position of the intermediate directly through steric or electrostatic effects, or indirectly influence orientation through subtle changes in the shape of the active site pocket. Cys372 also appears in the model to be close enough to Ser485 to possibly influence its nucleophilicity and thus indirectly influence catalysis. Both the C372S and F597W mutations shift the α- to β-pinene ratios toward α-pinene, and this could occur through diminished C7 deprotonation, improved C6 deprotonation, or a combination of these effects.

The other commonly observed result of reciprocal PS to CS mutations was an increase in the proportions of bornyl cation-derived products. These increases were very slight for single point mutations but the effects of multiple combined mutations were additive or, in some cases, synergistic. Synergistic mutational effects have also been reported for cycloartenol synthase from *Arabidopsis* [26]. In that enzyme, the I481V and Y401T mutations both result in alternative triterpenoid products, and a synergistic effect is observed in the double mutant. In the present study, it is unclear how the combination of mutations shift the reaction channel. The bornyl cation can arise either from rearrangement of the pinyl cation or through direct C1–C8 closure of the α-terpinyl cation (Fig. 3), and there is evidence for both routes amongst monoterpene synthases [40,41]. Some of the increase in bornyl cation-derived products may result from an increased steady-state level of the pinyl intermediate due to reduced C6 or C7 deprotonation. Because the bornyl cation route occurs to a small extent in wildtype PS, processes that reduce deprotonation of the pinyl intermediate could channel product through the alternative route. Some support for this proposal derives from non-reciprocal PS mutants (unpublished results), in which slightly increased proportions of camphene resulted. This simple "steady-state explanation," however, is probably not sufficient to account for the synergistic mutational effects observed here. The mutants in which the majority of product pass through the bornyl cation likely represent an introduced activity rather than a simple broadening of specificity that is often observed. Because the bornyl cation is energetically less favored, it is unlikely that the new activity represents broadening to a mechanistically simpler route. The structural underpinnings of this new activity, however, remain unclear.

The sum of all terpenoid synthase mutagenesis studies indicate that common enzymatic machinery is involved in producing the initial allylic cation intermediate but that the latter steps in the reaction proceed by a less defined mechanism in which the probability of a particular reaction channel is determined through the collective influence of residues both in and distant from the active site pocket. Fig. 5, which compares mutant #12-1 with wildtype PS, shows the wide spatial distribution of influential mutations. Some, such as C480S, S485C, and

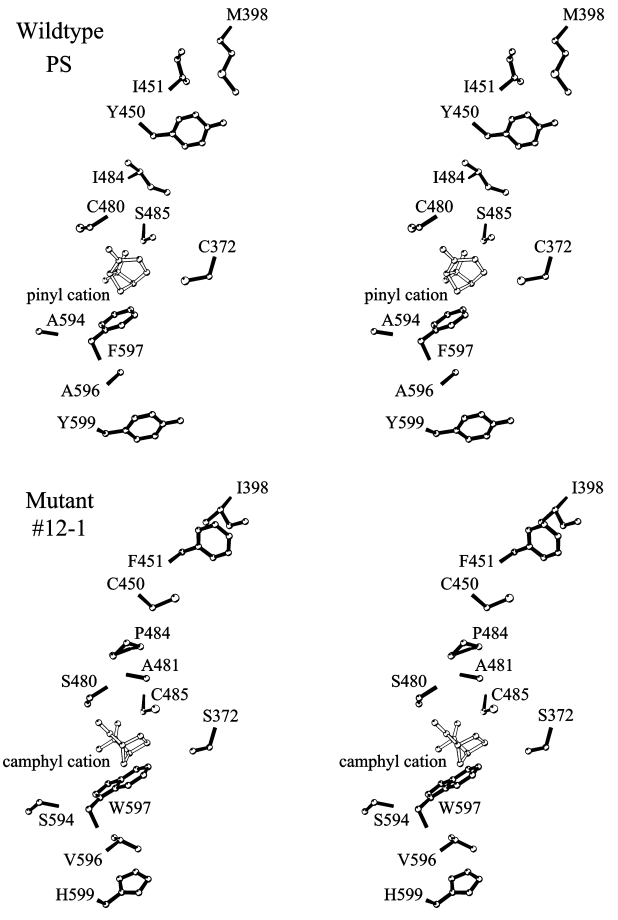

Fig. 5. Stereoviews of portions of the models of wildtype pinene synthase and mutant #12-1 (Table 2). The pinyl cation in the model of the wildtype enzyme is in the same position as in Fig. 4, and the camphyl cation is modeled in the same relative position in the model of the mutant enzyme.

F597W are located immediately in the predicted active site but several others are further away. These distant residues are too far removed to have a direct role in the reaction and are likely outside the range of electrostatic influence; yet, they collectively have a significant effect on product outcome. It was unexpected that a CS-like product profile did not result from complete exchange of the entire PS catalytic domain, because previous work with terpenoid synthases had shown that this region determines product outcome [13,15,16]. It appears that, in PS, even residues located outside the catalytic domain make some contribution to catalysis. It is possible that all influential residues collectively effect the average shape or dynamics of the active site. Future studies involving molecular dynamics simulations might thus be useful for understanding catalysis by enzymes of this class. The collective influence of several residues could explain both the difficulty in predicting mutational effects a priori and why the effects of single point mutations are often so subtle. It appears that a complete mechanistic understanding of PS and related terpenoid synthases will require studies that address the collective influence of a large number of amino acid residues.

References

[1] M.L. Wise, R. Croteau, in: D.E. Cane (Ed.), Comprehensive Natural Products Chemistry: Isoprenoids Including Carotenoids and Steroids, vol. 2, Elsevier Science, Oxford, 1999, pp. 97–153.

[2] D.E. Cane, in: D.E. Cane (Ed.), Comprehensive Natural Products Chemistry: Isoprenoids Including Carotenoids and Steroids, vol. 2, Elsevier Science, Oxford, 1999, pp. 155–200.

[3] J. MacMillan, M.H. Beal, in: D.E. Cane (Ed.), Comprehensive Natural Products Chemistry: Isoprenoids Including Carotenoids and Steroids, vol. 2, Elsevier Science, Oxford, 1999, pp. 217–243.

[4] K. Back, S. Yin, J. Chappell, Arch. Biochem. Biophys. 315 (1994) 527–532.

[5] C.L. Steele, J. Crock, J. Bohlmann, R. Croteau, J. Biol. Chem. 273 (1998) 2078–2089.

[6] E.M. Davis, R. Croteau, Top. Curr. Chem. 209 (2000) 53–95.

[7] K.U. Wendt, K. Poralla, G.E. Schultz, Science 277 (1997) 1811–1815.

[8] C.M. Starks, K. Back, J. Chappell, J.P. Noel, Science 277 (1997) 1815–1820.

[9] C.A. Lesburg, G. Zhai, D.E. Cane, D.W. Christianson, Science 277 (1997) 1820–1824.

[10] J.M. Caruthers, I. Kang, M.J. Rynkiewicz, D.E. Cane, D.W. Christianson, J. Biol. Chem. 275 (2000) 25533–25539.

[11] M.J. Rynkiewicz, D.E. Cane, D.W. Christianson, Proc. Natl. Acad. Sci. USA 98 (2001) 13543–13548.

[12] D.A. Whittington, M.L. Wise, M. Urbansky, R.M. Coates, R.B. Croteau, D.W. Christionson, Proc. Natl. Acad. Sci. USA 99 (2002) 15375–15380.

[13] K. Back, J. Chappell, Proc. Natl. Acad. Sci. USA 93 (1996) 6841–6845.

[14] T. Kushiro, M. Shibuya, Y. Ebizuka, J. Am. Chem. Soc. 121 (1999) 1208–1216.

[15] M.K. El Tamer, J. Lücker, D. Bosch, H.A. Verhoeven, F.W.A. Verstappen, W. Schwab, A.J. van Tunen, A.G.J. Voragen, R.A. de Maagd, H.J. Bouwmeester, Arch. Biochem. Biophys. 411 (2003) 196–203.

[16] R.J. Peters, R.B. Croteau, Arch. Biochem. Biophys. 417 (2003) 203–211.

[17] S. Katoh, D. Hyatt, R. Croteau, Arch. Biochem. Biophys. 425 (2004) 65–76.

[18] D.E. Cane, J.H. Shim, Q. Xue, B.C. Fitzsimons, Biochemistry 34 (1995) 2480–2488.

[19] K.A. Rising, C.M. Starks, J.P. Noel, J. Chappell, J. Am. Chem. Soc. 122 (2000) 1861–1866.

[20] B.M. Joubert, L. Hua, S.P.T. Matsuda, Org. Lett. 2 (1999) 339–341.

[21] J.B.R. Herrera, W.K. Wilson, S.P.T. Matsuda, J. Am. Chem. Soc. 122 (2000) 6765–6766.

[22] S.P.T. Matsuda, L.B. Darr, E.A. Hart, J.B.R. Herrera, K.E. McCann, M.M. Meyer, J. Pang, H.G. Schepmann, Org. Lett. 2 (2000) 2261–2263.

[23] M. Seemann, G. Zhai, J.-W. de Kraker, C.M. Paschall, D.W. Christianson, D.E. Cane, J. Am. Chem. Soc. 124 (2002) 7681–7689.

[24] D.B. Little, R.B. Croteau, Arch. Biochem. Biophys. 402 (2002) 120–135.

[25] R.J. Peters, R.B. Croteau, Proc. Natl. Acad. Sci. USA 99 (2002) 580–584.

[26] S. Lodeiro, M.J.R. Segura, M. Stahl, T. Schulz-Gasch, S.P.T. Matsuda, Chem. BioChem. 5 (2004) 1581–1585.

[27] E. Lewinsohn, M. Gijzen, R. Croteau, Plant Physiol. 96 (1991) 44–49.

[28] M. Gijzen, E. Lewsohn, R. Croteau, Arch. Biochem. Biophys. 289 (1991) 267–273.

[29] J. Bohlmann, C.L. Steele, R. Croteau, J. Biol. Chem. 272 (1997) 21784–21792.

[30] J. Bohlmann, M. Phillips, V. Ramachandiran, S. Katoh, R. Croteau, Arch. Biochem. Biophys. 368 (1999) 232–243.

[31] J. Bohlmann, J. Crock, R. Jetter, R. Croteau, Proc. Natl. Acad. Sci. USA 95 (1998) 6756–6761.

[32] B. Stofer Vogel, M.R. Wildung, G. Vogel, R. Croteau, J. Biol. Chem. 271 (1996) 23262–23268.

[33] P.M. Schenk, S. Baumann, R. Mattes, H.-H. Steinbiß, BioTechniques 19 (1995) 196–200.

[34] R. Croteau, W.R. Alonso, A.E. Koepp, M.A. Johnson, Arch. Biochem. Biophys. 309 (1994) 184–192.

[35] V.J. Davisson, A.B. Woodside, T.R. Neal, K.E. Stremler, M. Muehlbacher, C.D. Poulter, J. Org. Chem. 51 (1986) 4768–4779.

[36] T.A. Jones, J. Appl. Cryst. 11 (1978) 268–272.

[37] J. Sambrook, E.F. Fritsch, T. Maniatis, A Laboratory Mannual, second ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, 1989.

[38] W.R. Alonso, J.I.M. Rajaonarivony, J. Gershenzon, R. Croteau, J. Biol. Chem. 267 (1992) 7582–7587.

[39] R.P. Adams, Identification of Essential Oil Components by Gas Chromatography/Mass Spectroscopy, Allured Publishing, Carol Stream, Illinois, 1995.

[40] R.B. Croteau, C.J. Wheeler, D.E. Cane, R. Ebert, H.-J. Ha, Biochemistry 26 (1987) 5383–5389.

[41] K.C. Wagschal, H.-J. Pyun, R.M. Coates, R. Croteau, Arch. Biochem. Biophys. 308 (1994) 477–487.

[42] P.J. Kraulis, J. Appl. Crys. 24 (1991) 946–950.