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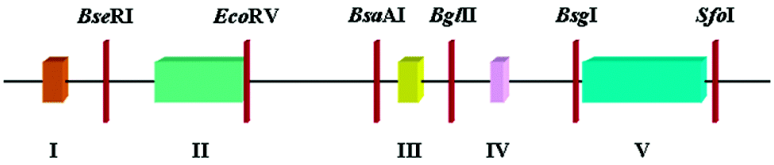

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Chrysanthemyl Diphosphate Synthase. The Relationship among Chain Elongation, Branching, and Cyclopropanation Reactions in the Isoprenoid Biosynthetic Pathway

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The vast majority of carbon skeletons in naturally occurring isoprenoid molecules are constructed by one of two "building" reactions, 1′−4 chain elongation and c1′−2−3 cyclopropanation. The chain elongation reaction attaches the hydrocarbon moiety of an allylic diphosphate to the double bond in isopentenyl diphosphate (IPP) to extend the chain by one isoprene unit (Scheme 1). A large group of enzymes catalyze chain elongation. Individual members are selective for the chain length of the allylic diphosphate substrate, the chain length of the final product, and the stereochemistry of the double bond introduced upon the addition of each molecule of IPP.[1,2] The chain elongation enzymes belong to one of two different structural classes that apparently evolved independently.[3,4] Farnesyl diphosphate (FPP) synthase is typical of those that synthesize isoprenoid chains with *E*-double bonds. The enzyme consists of α-helices arranged in a conserved "isoprenoid synthase" fold and catalyzes the synthesis of FPP from dimethylallyl diphosphate (DMAPP) by two successive additions of IPP.[3]

The cyclopropanation reaction joins two allylic diphosphates to produce a cyclopropylcarbinyl diphosphate. The most prominent enzymes that catalyze cyclopropanation are squalene synthase,[5,6] the first pathway-specific enzyme in sterol biosynthesis, and phytoene synthase,[7] the first pathway-specific enzyme in carotenoid biosynthesis. Under normal conditions, these enzymes catalyze two consecutive reactions − cyclopropanation and rearrangement of the cyclopropyl products.[8,9] The cyclopropylcarbinyl intermediates are not released during normal turnover,[10] and, as a result, metabolites with the carbon skeletons of presqualene and prephytoene diphosphate are not encountered in nature.

Sequence alignments suggest that squalene and phytoene synthases have similar structures. Although there is no discernible similarity between the amino acid sequences of the cyclopropanation enzymes and chain elongation enzymes, a recent X-ray structure of human squalene synthase revealed that the enzyme has the isoprenoid synthase fold seen in FPP synthase,[6] suggesting a common ancestor for the chain elongation and cyclopropanation enzymes. A third enzyme, chrysanthemyl diphosphate (CPP) synthase, catalyzes a related cyclopropanation between two molecules of dimethylallyl diphosphate (DMAPP) to give CPP, an intermediate in the biosynthesis of the naturally occurring pyrethrin insecticides in chrysanthemums.[11] In this case, CPP is the final product of the synthase. Although CPP synthase catalyzes cyclopropanation, its amino acid sequence has high similarity to FPP synthase, indicative of the recent evolution of its cyclopropanation activity from ancestral chain elongation.

The chain elongation reaction proceeds by a dissociative electrophilic alkylation of the double bond in IPP by the allylic substrate. Although several mechanisms have been proposed for the cyclopropanation reaction, there is little direct evidence to support any of these proposals. However, the high degree of similarity between the amino acid sequences of FPP synthase and CPP synthase

Scheme 1

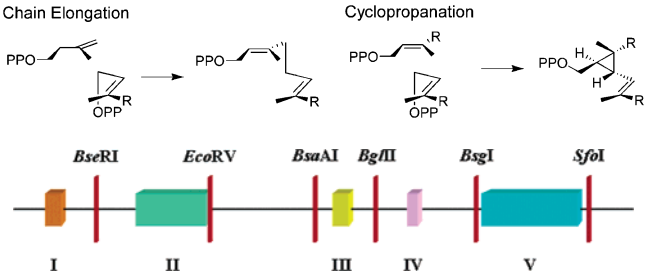

Figure 1. Schematic representation of the orfs for sage FPP and CPP synthases where the boxes represent the five conserved regions found in the *E*-chain elongation prenyl transferases. The vertical red lines define the location of the restriction sites used to construct chimeras.

suggests that chain elongation and cyclopropanation may proceed by similar mechanisms. We have recently isolated and characterized open reading frames for FPP synthase and CPP synthase from a cDNA library from *Artemisia tridentata speciformis*, a species of sagebrush that grows at higher altitudes in the Great Basin of the western United States.[12] The amino acid sequences of the encoded proteins were 75% identical. We now report studies with chimeric proteins constructed from segments of sage CPP and FPP synthase, which provide strong evidence that the cyclopropanation reaction is an electrophilic alkylation.

The prenyltransferases that catalyze *E*-double bond chain elongation have five conserved regions (I−V, see Figure 1) which form the active site in the enzymes.[13] All but a few of the amino acids in these regions are identical in FPP synthase and CPP synthase from sage. Open reading frames (orfs) for sage FPP and CPP synthases, each containing 3′ extensions encoding six histidines followed by a factor Xa recognition site, were subcloned into pUC19 (New England Biolabs). *Bse*RI, *Eco*RV, *Bgl*II, *Bsg*I, and *Sfo*I restriction sites were introduced into the orf of sage CPP synthase, and *Bse*RI, *Bsa*AI, *Bsg*I, and *Sfo*I sites were introduced into the orf of sage FPP synthase to generate, in combination with natural restriction sites, six common restriction sites at identical locations in the two orfs. These constructs encoded mutant versions of FPP synthase (FPP-M synthase with mutations G69E and E210Q) and CPP synthase (CPP-M synthase with mutations M98I, E177D, and D243A). The mutations did not significantly alter kinetic constants for the two enzymes. The mutant orfs were used to construct fusions that encoded six chimeric proteins, in which amino acids in FPP-M synthase, beginning at the N-terminus, were progressively replaced with the corresponding residues from CPP-M synthase. The chimeras are named according to the first three letters of the restriction site used to fuse sequences from FPP-M synthase and CPP-M synthase (Figure 1). The orfs were each subcloned into pMPM3B and expressed in *E. coli*. The resulting proteins were purified (>95%) by Ni^{2+} affinity chromatography.

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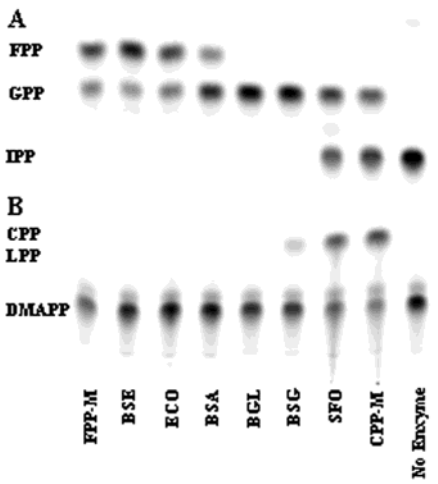


Figure 2. Phosphor audioradiograms of chain elongation (A) and cyclopropanation (B) assays. ^{14}C labeled samples of IPP and DMAPP are given in lanes 9 (A) and 9 (B), respectively. Lanes in part A show ^{14}C labeled products obtained from FPP-M, BSE, ECO, BSA, BGL, BSG, SFO, and CPP-M, respectively, when incubated with [^{14}C] IPP and DMAPP. Lanes in part B show ^{14}C labeled products for the same enzymes when incubated with [^{14}C] DMAPP.

FPP-M synthase, CPP-M synthase, and the six chimeras were screened for chain elongation and cyclopropanation activity. The substrates for chain elongation were [^{14}C]IPP and DMAPP,[14] while [^{14}C]DMAPP was the substrate for cyclopropanation.[15] Reactions were initiated by the addition of the appropriate enzyme (12.5 μM final concentration), and samples were incubated at 30 °C for 2 h before being quenched with 50 μL of ethanol. The reaction mixtures were spotted on silica gel TLC plates and developed with 30:70:16:10 $CHCl_3$:pyridine:formic acid:water. The products were visualized by phosphor autoradiography (Figure 2).

When analyzed for chain elongation, FPP-M synthase efficiently produced [^{14}C]FPP from DMAPP and two molecules of [^{14}C]IPP (Figure 2A). A small amount of the C_{10} intermediate [^{14}C]geranyl diphosphate (GPP) was also detected. The first two chimeras, BSE and ECO, gave essentially identical results (Figure 2A). The third chimera in the series, BSA, also produced [^{14}C]GPP and [^{14}C]FPP, but the second addition of IPP was substantially slower, and [^{14}C]-GPP was now the major product. The next two chimeras, BGL and BSG, were highly selective for the synthesis of [^{14}C]GPP. SFO and CPP-M synthase also synthesized [^{14}C]GPP, although less efficiently than the other enzymes. The transition from FPP synthase to GPP synthase activity was first evident for BSA, where regions I and II in FPP-M synthase were replaced by the corresponding residues from CPP-M synthase. Region II in the chain elongation enzymes contains a highly conserved aspartate-rich DDxxD motif responsible for binding the diphosphate moiety of the allylic substrate and an α-helix that is important for regulating the ultimate chain length of the product.[1,2] The selectivity for the synthesis of GPP increased dramatically in the BGL enzyme upon substitution of region III by sequenes from CPP-M synthase. Thus, it appears that the residues in this region are also important for determining chain length.

FPP-M synthase and the chimeras with CPPaseM replacements through BGL were inactive in the cyclopropanation assay. It was only for BSG, when regions I−IV of FPP-M synthase had been replaced with the corresponding sequence from CPP-M synthase in BSG, that a small amount of product was seen in the cyclopropanation assay (Figure 2B, lane 6). The last chimera in the series, SFO, only contained a C-terminal fragment after region V from FPP-M synthase and appeared to be similar to CPP-M synthase in the cyclopropanation assay. Cyclopropanation activity was not seen until the first four conserved regions in FPP-M synthase had been replaced. The final conserved region, V, contains a second DDxxD motif responsible for IPP binding in chain elongation. It was only after the replacement of region V that a substantial enhancement was seen in cyclopropanation activity.

Table 1. Ratios of Cyclopropanation, Branching, and Chain Elongation[a]

synthase	DMAPP COH:LOH	DMAPP + IPP COH:LOH:GOH
CPP	3.6 ± 0.1	3.5 ± 0.1: 1: 0.96 ± 0.04
CPP-M	2.1 ± 0.1	2.1 ± 0.1: 1: 1.0 ± 0.1
SFO	0.41 ± 0.01	0.41 ± 0.01: 1: 0.6 ± 0.04
BSG	LOH only	GOH only

[a] Ratios of alcohols corresponding to the isoprenoid diphosphates produced by the enzymes in the presence of DMAPP or both DMAPP and IPP. See the Supporting Information for details.

To verify the identities of the diphosphate products detected in the chain elongation and cyclopropanation assays, incubations were conducted with unlabeled substrates, the diphosphate moieties were removed by treatment with alkaline phosphatase, and the resulting alcohols were analyzed by gas chromatography/mass spectrometry (GCMS). As expected, geraniol (GOH) and farnesol (FOH) were obtained from reactions conducted under chain elongation conditions. However, analysis of the hydrolysis products from incubation of DMAPP with CPP-M synthase revealed the presence of two alcohols, the cyclopropanation product, chrysanthemol (COH), and a monoterpene alcohol, lavandulol (LOH), produced by a 1′−2 coupling of the isoprenoid units in DMAPP.[12] Although the corresponding diphosphates, CPP and lavandulyl diphosphate (LPP), were formed from DMAPP in the chain elongation assay and cochromatograph with GPP, the compounds were not radioactive and do not contribute to the signal for GPP during phosphorimaging.

The relative amounts of COH, LOH, and GOH obtained under chain elongation and cyclopropanation conditions were determined for all of the enzymes and are given in Table 1. As expected, they all gave GOH. BSG was the first enzyme to show activity in the cyclopropanation assay. However, the only monoterpene alcohol obtained after hydrolysis of the C_{10} diphosphate was LOH! The appearance of LOH coincided with substitution of conserved regions I−IV in FPP-M synthase by CPP-M synthase. The next chimera in the series, SFO, gave both COH and LOH in a ratio of 0.4:1. This ratio increased to 2.1:1 for CPP-M synthase and to 4.2:1 for wild-type CPP synthase. The slightly lower ratio observed for CPP-M synthase is probably due to the D247A mutation introduced by incorporation of the *Sfo*I restriction site. It seems less likely that the two other mutations in CPP-M synthase, M101I and E177D, are responsible for altering the CPP/LPP ratio because the closely related CPP synthase from *Chrysanthemum cinerafolium* contains leucine and aspartate residues at these respective positions and gave a CPP/LPP ratio of ∼4:1. The synthesis of CPP was only seen after all five conserved regions in FPP-M synthase had been replaced. Region V contains the highly conserved DDxxD motif responsible for binding IPP in the chain elongation enzymes and presumably the molecule of DMAPP that serves as the prenyl acceptor in the cyclopropanation reaction.

The rates for chain elongation, cyclopropanation, and branching were measured at the same fixed substrate concentrations used for the corresponding product studies shown in Figure 2.[14,15] As seen in Table 2, the rate of chain elongation decreased by ∼4000-fold as FPP-M was transformed into CPP-M. No cyclopropanation-branching activity was detected for FPP-M. BSG was the first enzyme in the series where activity was detected with DMAPP.

Table 2. Rates for Chain Elongation and Cyclopropanation-Branching[a]

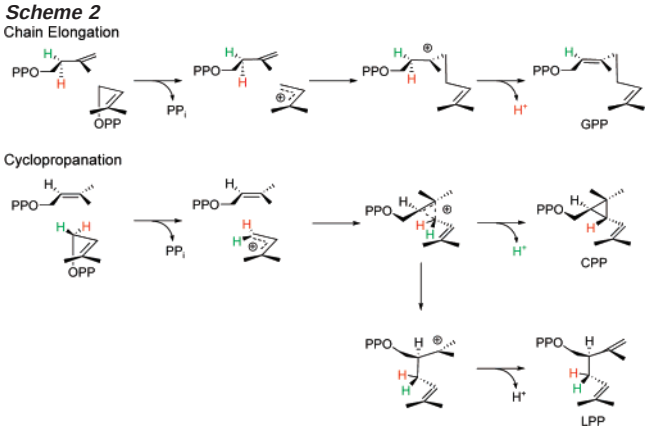
k_{cat}^{app} (s^{-1})	FPP-M	BSG	CPP-M
elongate	3.7 ± 0.1	0.045 ± 0.002	0.001 ± 0.0003
cyclopropanate-branch	no reaction	0.0008 ± 0.00005	0.03 ± 0.003

[a] Experimental procedures are described in the Supporting Information.

At this point, all of the amino acids in FPP-M except those from the beginning of the conserved region V to the C-terminus had been replaced by amino acids from CPP-M. LPP was the only product from DMAPP. As the final amino acids were replaced to give CPP-M, the DMAPP activity increased ~40-fold. For the substrate concentrations used in the chain elongation assay, the cyclopropanation-branching activity was ~4.8-fold greater than that of chain elongation.

Our results clearly demonstrate that the catalytic machinery for chain elongation of DMAPP to FPP via GPP by two successive additions of IPP can be transformed, with rather minimal changes, into the machinery necessary for the synthesis of CPP from two molecules of DMAPP. There is a transition from chain elongation to cyclopropanation activity as peptide sequences in FPP synthase are replaced with the corresponding regions in CPP synthase that can be divided into two stages. During the first stage, corresponding to substitution of amino acids 1−176, the binding pocket for allylic substrate is altered to prevent binding of GPP. This blocks the second chain elongation reaction and prevents the synthesis of FPP. In the second stage, beginning with amino acid 224, the IPP binding site is modified to accommodate DMAPP. At first, LPP is produced, and then, after fine-tuning, CPP. The chain elongation reaction catalyzed by FPP synthase is a dissociative electrophilic alkylation of the double bond in IPP by the allylic cations generated from DMAPP and GPP.[16,17] The enzyme catalyzes chain elongation exclusively by rigorously excluding DMAPP from the IPP binding region.[18] As the IPP site is modified to permit binding of DMAPP, a similar reaction occurs to produce LPP. The mechanistic similarity between the branching and chain elongation reactions is obvious. Further refinement results in the formation of the cyclopropane ring in CPP by insertion of C(1) from one molecule of DMAPP into the C2−C3 double bond of another. This reaction is a logical extension of the branching reaction, where DMAPP bound in what was originally the "IPP" site is alkylated by a dimethylallyl cation to form a protonated cyclopropane, which subsequently loses a proton to give CPP (see Scheme 2). While it is not possible to know if the allylic region of the active site of FPP synthase was modified before the IPP binding site during the evolution of CPP synthase from FPP synthase in higher plants, it is a reasonable scenario and is consistent with the absence of non-head-to-tail sesquiterpenes formed from condensation of GPP with DMAPP in sage. Phylogenetic considerations suggest that the cyclopropanation activity seen for squalene/photoene synthase first evolved from a chain elongation enzyme at the very beginning of cellular evolution.

Scheme 2



Its recent reemergence in CPP synthase has provided crucial insights about the mechanism of the reaction.

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Supporting Information Available: Procedures for plasmid construction, protein purification, and product analysis. Amino acid sequence alignments for sage FPP and CPP synthase (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

References

(1) Kellog, B. A.; Poulter, C. D. *Curr. Opin. Chem. Biol.* **1997**, *1*, 580−585.
(2) Ogura, K.; Koyama, T. *Chem. Rev.* **1998**, *98*, 1263−1274.
(3) Tarshis, L. C.; Yan, M.; Poulter, C. D.; Sachettini, J. C. *Biochemistry* **1994**, *33*, 10871−10877.
(4) Fujihashi, M.; Zhang, Y.-W.; Higuchi, Y.; Li, X.-Y.; Koyama, T.; Miki, K. *Proc. Natl. Acad. Sci. U.S.A.* **2001**, *98*, 4337−4342.
(5) Zhang, D. L.; Jennings, S. M.; Robinson, G. W.; Poulter, C. D. *Arch. Biochem. Biophys.* **1993**, *304*, 133−143.
(6) Pandit, J.; Danley, D. E.; Schulte, G. K.; Mazzalupo, S.; Pauly, T. A.; Hayward, C. M.; Hamanaka, E. S.; Thompson, J. F.; Harwood, H. J. *J. Biol. Chem.* **2000**, *39*, 30610−30617.
(7) Iwata- Reuyl, D.; Math, S. K.; Desai, S. B.; Poulter, C. D. *Biochemistry* **2003**, *42*, 3359−3365.
(8) Jarstfer, M. B.; Zhang, D. L.; Poulter, C. D. *J. Am. Chem. Soc.* **2002**, *124*, 8834−8845.
(9) Blagg, B. S. J.; Jarfster, M. B.; Rogers, D. H.; Poulter, C. D. *J. Am. Chem. Soc.* **2002**, *124*, 8846−8853.
(10) Radisky, E. S.; Poulter, C. D. *Biochemistry* **2000**, *39*, 4096−4104.
(11) Rivera, S. B.; Swedlund, B. D.; King, G. J.; Bell, R. N.; Hussey, C. E.; Shattuck-Eidens, D. M.; Wrobel, W. M.; Peiser, G. D.; Poulter, C. D. *Proc. Natl. Acad. Sci. U.S.A.* **2001**, *98*, 4373−4398.
(12) Unpublished results.
(13) Chen, A.; Kroon, P. A.; Poulter, C. D. *Protein Sci.* **1994**, *3*, 600−607.
(14) 140 μM DMAPP and 50 μM [^{14}C]IPP (50 μCi/μmol) in 35 mM HEPES buffer, pH 7.6, containing 10 mM $MgCl_2$ and 4 mM DTT in a total volume of 20 μL.
(15) 400 μM [^{14}C]DMAPP (20 μCi/μmol) in 35 mM HEPES buffer, pH 7.6, containing 10 mM $MgCl_2$ and 4 mM DTT in a total volume of 20 μL.
(16) Poulter, C. D.; Wiggins, P. L.; Le, A. T. *J. Am. Chem. Soc.* **1981**, *103*, 3926−3927.
(17) Davisson, V. J.; Neal, T. R.; Poulter, C. D. *J. Am. Chem. Soc.* **1993**, *115*, 1235−1245.
(18) Rilling, H. C. *Pure Appl. Chem.* **1979**, *51*, 897−608.

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