Functional Analysis of the Two Interacting Cyclase Domains in ent-Kaurene Synthase from the Fungus Phaeosphaeria sp. L487 and a Comparison with Cyclases from Higher Plants*

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We report here kinetic analysis and identification of the two cyclase domains in a bifunctional diterpene cyclase, Phaeosphaeria ent-kaurene synthase (FCPS/KS). Kinetics of a recombinant FCPS/KS protein indicated that the affinity for copalyl diphosphate is higher than that for geranylgeranyl diphosphate (GGDP). ent-Kaurene production from GGDP by FCPS/KS was enhanced by the addition of a plant ent-kaurene synthase (KS) but not by plant CDP synthase (CPS), suggesting that the rate of ent-kaurene production of FCPS/KS may be limited by the KS activity. Site-directed mutagenesis of aspartate-rich motifs in FCPS/KS indicated that the affinity for copalyl diphosphate (CDP) is lower than that for geranylgeranyl diphosphate (GGDP); CDP, (-)-copalyl diphosphate; CPS, copalyl-diphosphate synthase; SHC, squalene-hopene cyclase; GST, glutathione S-transferase; CT, C-terminal truncation; FCPS/KS, fungal-type ent-kaurene synthase; KS, ent-kaurene synthase; NT, N-terminal truncation.

ent-Kaurene (Fig. 1, 3) is a tetracyclic diterpene hydrocarbon and one of the biosynthetic intermediates in the production of the gibberellin (GA)* plant hormones. Both higher plants and GA-producing fungi produce ent-kaurene from geranylgeranyl diphosphate (GGDP, 1) via (-)-copalyl diphosphate (CDP, 2) (1). In higher plants, two different diterpene cyclases, copalyl-diphosphate synthase (CPS) and ent-kaurene synthase (KS), are involved in the biosynthesis of ent-kaurene; CPS catalyzes the cyclization of GGDP to CDP and KS catalyzes the cyclization of CDP to ent-kaurene (2). To date, several CPS and KS genes have been cloned from higher plants (3–8). These genes encode proteins with N-terminal transit peptides that the enzymes target to the plastid (3, 9, 10). Recently, an ent-kaurene synthase cDNA has been cloned from a GA-producing fungus Phaeosphaeria sp. L487, and the function of the gene investigated. In contrast to plant enzymes, the fungal ent-kaurene synthase (FCPS/KS) catalyzes the two-step cyclization reaction from GGDP to ent-kaurene via CDP (11). This FCPS/KS is a bifunctional diterpene cyclase having both activities of plant CPS and KS.

Conversion of GGDP to ent-kaurene in fungi and plants involves two different cyclization reactions (12–14). According to cyclization mechanisms and aspartate-rich motifs, diterpene cyclases are classified into two groups by MacMillan and Beale (15). Type A cyclization reaction is initiated by ionization of the diphosphate, and type B cyclization reaction is initiated by protonation at the 14,15-double bond of GGDP. The reaction of CPS is initiated as a type B cyclization and proceeds without rearrangement of the carbon skeleton (Fig. 1). A similar cyclization reaction, catalyzed by squalene-hopene cyclase (SHC) from Ailicyclobacillus acidocaldarius, is initiated by protonation of the carbon-carbon double bond of squelene (16, 17). In 1997, x-ray crystallographic analysis of recombinant SHC revealed that the aspartate-rich DVDDTA motif (motif b) is responsible for initiation of cyclization (18). It is believed that the second aspartate residue acts as the proton donor. This hypothesis is supported by loss of activity in SHC mutants in which the aspartate-rich motif was exchanged by site-directed mutagenesis (19). In contrast to CPS, cyclization of KS, type A cyclization, is initiated by elimination of the diphosphate group of CDP and is accompanied by rearrangement of the carbon skeleton (Fig. 1). This mechanism is shared by a number of plant mono-, sesqui- and diterpene cyclases (20).

The aspartate-rich DDXD motif (motif a) in terpen synthase is believed to be one of the sites to which the substrate is bound via divalent metal ion bridges (21). This motif, which is conserved in both prenyl transferases and terpene cyclases, has been characterized by site-directed mutagenesis (22–26). In 1997, 5-epi-aristolochene synthase and pentalenene synthase cloned from tobacco (27) and an actinomycete (28), respectively, were analyzed by x-ray crystallography (29, 30). The analyses revealed that the function of the first aspartate residue in DDXD is to bind substrate via a magnesium ion.

We showed previously that the CPS activity of FCPS/KS is inhibited by the quaternary ammonium reagent Amo-1618, whereas KS activity was unaffected (11). These data suggest that FCPS/KS may have separate catalytic domains for the two-step cyclization reaction in the single polypeptide chain. The N-terminal region of FCPS/KS shows similarity with many diterpene cyclases including CPS and KS. An aspartate residue of motif b, which is a putative proton donor in FCPS/KS, is

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§ The abbreviations used are: GA, gibberellin; GGDP, geranylgeranyl diphosphate; CDP, (-)-copalyl diphosphate; CPS, copalyl-diphosphate synthase; SHC, squalene-hopene cyclase; GST, glutathione S-transferase; CT, C-terminal truncation; FCPS/KS, fungal-type ent-kaurene synthase; KS, ent-kaurene synthase; NT, N-terminal truncation.

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Preparation of Deletion Clones—The antisense primers used to construct the CT-513, CT-530, CT-568, and CT-900 mutants were 5'-AG-GCC-TGCGG-GCGTCTAGCCTGC-3', 5'-TGGCTGAGTGGCCG-TTTGGAGCT-CA-3', 5'-ACCCGTCGAGCTGCTGAAACC-CTTG-3', and 5'-ACCCGTCGAGGGGAGATTGAAAGAACCCT-3', respectively. The sense primer for CT mutants was 5'-CGATGATGCTATGCTGGCCAAATTTGATATCC-3'. The sense primers used to construct the NT-44, -250, -359, and -382 mutants were 5'-CGATGATGCTATGCTGGCCAAAGAGGACC-3', 5'-CGATGATGCTATGCTGGCCAAAGAGGACC-3', 5'-CGATGATGCTATGCTGGCCAAAGAGGACC-3', and 5'-CGATGATGCTATGCTGGCCAAAGAGGACC-3', respectively. The antisense primer for the NT mutants was 5'-GGCCCGTCGAGCTUCATAGGTCCGCAG-3'. Sense and antisense primers contained restriction enzyme sites for BamHI and XhoI (underlined) at each 5'-end. Polymerase chain reaction-amplified DNA fragments were digested with BamHI and XhoI and ligated into a pGEX4T-3 vector. The DNA sequences of deletion clones were confirmed by sequencing.

Oxidoreduction and Purification of GST-fused Proteins—Procedures were based on protocols described elsewhere (33). A transformed E. coli strain BL21(DE3)pLysS harboring a plasmid for protein expression was cultured in 2× YT medium supplemented with ampicillin (100 μg ml⁻¹) and 0.1% (w/v) glucose at 30 °C for 6 h. After cooling the culture broth on ice, a recombinant GST-fused protein was produced by a 20-h incubation at 20 °C after the addition of 0.6 mM isopropyl-β-D-galactopyranoside. E. coli cells were collected and resuspended in a lysis buffer: 50 mM Tris-HCl, (pH 8.0), 1 mM dithiothreitol, 0.5 mM EDTA, 10% (v/v) glycerol, 0.5 mM MgCl₂, and 0.1% (w/v) glucose. After the freezing and thawing treatments, cells were disrupted by brief sonication. The soluble protein fraction was obtained by centrifugation (12,000 × g, 40 min). According to the manufacturer’s instruction, recombinant GST-fused proteins were purified by affinity chromatography using GST resin (glutathione-Sepharose 4B, Amersham Pharmacia Biotech) and obtained as the single bands on SDS-polyacrylamide gel electrophoresis. The FCPS/KS protein was purified 128-fold from crude cell extracts (252 mg) from 1 liter of culture by the chromatography to yield 731 μg of purified protein. Protein concentration was determined by protein-dye standard assay (Bio-Rad) using bovine serum albumin as a standard.

In Vitro Cyclase Assay—Cyclase activity from [³H]GGDP was measured by the production of ent-kaurene and purified on silica gel TLC (Kieselgel 60, Merck) developed with n-hexane, according to Saito et al. (34). A standard assay was performed by incubating 3.3 kBq of [³H]GGDP (55.5 GBq mmol⁻¹) and 1.6 kBq of [³H]CDP with 2 μg of purified FCPS/KS or mutant proteins in 50 mM Tris-HCl (pH 8.0) containing 5 mM dithiothreitol, 0.5 mM EDTA, 10% (v/v) glycerol, and 5 mM MgCl₂ at 30 °C for 15 min. To measure individual CPS or KS activity, 2 μg of GST-fused CmCPS or CmKS protein were added to the incubation in a standard assay mixture, and ent-kaurene production was measured as before. To identify products converted from GGDP, nonlabeled GGDP was incubated with recombinant GST-fused proteins and analyzed by full-scan gas chromatography-mass spectrometry as described previously (11). Kinetic analyses were performed by determination of the ent-kaurene production using [³H]GGDP and [³H]CDP as substrates. To determine parameters for GGDP, [³H]GGDP was incubated with 2 μg of FCPS/KS or CmCPS at 30 °C for 15 min. After termination of the reaction, CmKS (4 μg) was added to the reaction mixture to convert CDP to ent-kaurene. The Michaelis constants (Kₘ) were determined from double-reciprocal plots.

RESULTS

Characteristics of Wild-type FCPS/KS for ent-Kaurene Production—To compare the mechanisms of ent-kaurene production between fungi and plants, the kinetic parameters of FCPS/KS were determined using purified proteins. [¹³C]GGDP and [¹⁵³C]CDP were used as substrates. Table II showed kinetic parameters for recombinant GST-fused FCPS/KS, CmCPS, and CmKS proteins. Relative cyclase activity was

| Mutant | Motifs | Site-directed mutagenesis of D/E-rich motifs in FCPS/KS |
|--------|--------|--------------------------------------------------------|
| Ala¹³²  | DDVLD  | 394GAC → 394GCC                                       |
| Ala²²⁰  | DVYDD  | 956GAC → 956GCC                                       |
| Ala⁵⁵⁶  | DEFFE  | 1966GAC → 1966GCC                                     |
shown as \( k_{\text{cat}} K_m^{-1} \). The \( K_m \) value of FCPS/KS for GGDP was 38-fold higher than that for CDP, indicating that the enzyme has a higher affinity for CDP than for GGDP. Although the \( k_{\text{cat}} \) value of FCPS/KS for GGDP was 26-fold higher than that for CDP, the relative KS activity (\( k_{\text{cat}} K_m^{-1} \)) of FCPS/KS seemed slightly higher than the CPS activity. The \( K_m \) values of CmCPS for GGDP and of CmKS for CDP were higher than those of FCPS/KS. Taking the relative activities into account, the relative activity of CmKS was 2.6-fold higher than that of CmCPS.

Interestingly, high substrate concentrations (10–13 \( \mu \text{M} \)) of GGDP inhibited the CPS activity of CmCPS, whereas no such substrate inhibition was observed in FCPS/KS in the same range of GGDP concentrations (Fig. 2). No inhibition was observed in FCPS/KS and CmKS in the high substrate concentrations of CDP.

**Site-directed Mutagenesis Study**—To study the role of the aspartate-rich motifs and found in FCPS/KS, three asparagine residues were substituted by the nonpolar residue, alanine (A). The recombinant mutated proteins were produced in E. coli, purified by the same procedure as the wild-type FCPS/KS protein, and used for cyclase assay. Mutant Ala\(^{132}\) produced ent-kaurene from GGDP and CDP, although with reduced efficiency (Table III). The \([\text{Ala}^{129}]^{-}\) and \([\text{Ala}^{656}]^{-}\) proteins produced no ent-kaurene, suggesting that these proteins lacked either CPS or KS activity. To confirm which activity was lacked, incubation of \([^{3}\text{H}]\text{CDP}\) with these mutated proteins was tested. The \([\text{Ala}^{129}]^{-}\) protein showed KS activity when incubated with \([^{3}\text{H}]\text{CDP}\), but the \([\text{Ala}^{656}]^{-}\) protein had no such activity, indicating that Asp\(^{320}\) is involved in the CPS reaction. In contrast, ent-kaurene production by Ala\(^{656}\) was observed when CmKS was added to the incubation, suggesting that the \([\text{Ala}^{656}]^{-}\) protein has CPS activity and that mutation at Asp\(^{320}\) affects the KS active domain. Interestingly, addition of CmKS into the solution containing the wild-type protein or mutant Ala\(^{132}\) resulted in increased ent-kaurene production. When \([^{3}\text{H}]\text{GGDP}\) was incubated with CmCPS and CmKS together under the same conditions, the levels of ent-\([^{3}\text{H}]\)kaurene production showed similar to that of the wild-type protein in the presence of CmKS. However, addition of CmCPS to the reaction mixtures containing the wild-type or \([\text{Ala}^{132}]^{-}\) protein did not enhance ent-\([^{3}\text{H}]\)kaurene production.

**Identification of Two Cyclase Domains in FCPS/KS**—A functional assay using a GA biosynthetic inhibitor (11) and site-directed mutagenesis suggested that FCPS/KS may have two catalytic sites for the cyclization reaction. To demonstrate that there are two functional cyclase domains, truncated polypeptides of FCPS/KS were prepared, and their cyclase activities were analyzed. Fig. 3 showed N-terminal and C-terminal truncated proteins of FCPS/KS. The mutant proteins were produced as GST-fused protein in E. coli. Cyclase activity of each mutants was estimated by production of ent-\([^{3}\text{H}]\)kaurene using \([^{3}\text{H}]\text{GGDP}\) as substrate. None of the mutant proteins could produce hydrocarbon compounds such as ent-kaurene from GGDP. Even GST-fused proteins NT-44 and CT-900, which are 127-kDa polypeptides and lack 43 and 46 amino acid residues from the N and C terminus of the wild-type, respectively, showed no bifunctional activity. After the addition of CmKS to the reaction mixture of the CT-530 (85 kDa), CT-568 (89 kDa), or CT-900 protein, ent-kaurene production was observed, suggesting that these mutants may have CPS activity. The CT-513 (83 kDa) protein showed no CPS activity. In contrast, the addition of CmCPS to the reaction mixture of NT-250 (103 kDa) or NT-359 (92 kDa) protein resulted in the production of a hydrocarbon compound identified as ent-kaurene (see below in the case of NT-359). The NT-382 (89 kDa) protein obtained by further truncation of the N-terminal region to NT-359 protein lacked any cyclase activities and did not produce a hydrocarbon compound with the addition of CmCPS. The NT-44 protein showed KS-like activity in the presence of CmCPS. Although the polypeptide length of NT-44 is longer than NT-359, the activity of the NT-44 protein was weaker than that of the NT-359 protein. NT mutants smaller than the NT-382 protein did not have any cyclase activities (data not shown).

To identify products converted from GGDP or CDP by truncated proteins, these substrates were incubated with truncated proteins, and the products were analyzed by gas chromatography-mass spectrometry. The CT-530 and CT-568 proteins converted GGDP to CDP identified as copalol (peak at 8:27; \( m/z \) 290 ([M]\(^+\), 6% relative intensity), 275 (56), 257 (76), 243 (6), and 95 (100)) but not to ent-kaurene. The NT-359 protein converted CDP to ent-kaurene (peak at 6:59 min; \( m/z \) 272 ([M]\(^+\), 58), 257 (100), 243 (2), and 229 (80)). These truncated proteins did not produce other diterpene metabolites.
Our studies using recombinant GST-fused proteins have revealed differences in the enzymes for ent-kaurene biosynthesis in fungi and higher plants. The CPS activity of CmCPS was inhibited at high concentration of the substrate GGDP (Fig. 2). This result is in agreement with characteristics of a native CPS from Marah macrocarpus (35). However, no substrate inhibition occurred in FCPS/KS. The synthesized ent-kaurense is turned over rapidly, and its concentration is usually very low in plants (36, 37). It is reported that ent-kaurene biosynthesis in pumpkin may be regulated by CPS genes rather than the KS gene, because CPS genes showed organ-specific expression patterns (6). The mRNA levels of KS are higher than those of the CPS gene and did not change during developmental stages (6, 10). In addition, GGDP is the major intermediate of isoprenoid biosynthesis in plastids and is synthesized to serve as an intermediate for GAs, carotenoids, chlorophylls, and other diterpene compounds in plants (13, 38). If substantial amounts of GGDP are produced in plastids of plants, ent-kaurene production leading to GA biosynthesis may not only be regulated by CPS gene transcript levels (39) but also GGDP levels. In contrast to CPS, the activity of plant diterpene cyclases like abi-1 pene compounds in plants (13, 38). If substantial amounts of GGDP are produced in plastids of plants, ent-kaurene production leading to GA biosynthesis may not only be regulated by CPS gene transcript levels (39) but also GGDP levels. In contrast to CPS, the activity of plant diterpene cyclases like abi-

**DISCUSSION**

We observed the fact that ent-kaurene production by FCPS/KS is enhanced by the addition of CmKS but not by CmCPS (Table III). The relative KS activity of CmKS seems higher than that of FCPS/KS (Table II), although the affinity of FCPS/KS for CDP is higher than that of CmKS. The levels of ent-[^3H]kaurene production were similar to that of incubation with CmCPS and CmKS together. This enhancement may be caused by KS activity of CmKS and increase of CPS activity of FCPS/KS induced by the addition of CmKS. The addition of CmCPS into the FCPS/KS mixture did not change the level of ent-kaurene production, indicating that CmCPS does not change the rate of KS activity of FCPS/KS. These results suggested that the rate of ent-kaurene production by FCPS/KS is limited by the KS activity.

We demonstrated that FCPS/KS has two functional cyclase domains with CPS and KS activity in the single polypeptide chain. We identified the CPS and KS active domains, which are located in tandem from the N-terminal to the C-terminal region in FCPS/KS. The CPS active domain with 63 kDa shares significant homology with both type A cyclases and type B cyclases (15). Site-directed mutagenesis of FCPS/KS showed the putative active sites for the two cyclase domains. Although little data are available on site-directed mutagenesis of type B cyclases, the Asp-320 residue in the 318DVDD motif is involved in the CPS reaction and probably plays a similar role to that of SHC (19). The x-ray analysis of A. acidocaldarius SHC proposed that amino acid residues of Ghu-45, Ghu-93, Asp-127, Gln-262, and His-451 are characterized as elements of the catalytic center (18). However, there is no significant homology between FCPS/KS and SHC, and these amino acid residues of SHC are not conserved in FCPS/KS. Therefore, except for the DXDD motif, it has been difficult to characterize structural properties of the domain.

In contrast to the CPS active domain, the KS active 66-kDa domain of FCPS/KS shows no significant homology with terpene cyclases initiated by the release of the diphosphate group of KS. Site-directed mutagenesis of the Asp-656 residue lacks KS activity (Table III), indicating that the 650DEFFE motif is indispensable for forming catalytic sites in the KS active domain. This may have the same function as the DDXXD motif.

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**TABLE III**

| Name  | Standard[^H]GGDP |[^H]CDP | + CmCPS[^a] | + CmKS[^b] | Cyclase activity (pmol - mg of protein)^-1 min^-1 |
|-------|------------------|--------|-----------|-----------|-----------------------------------------------|
| WT    | 750 ± 48         | 238 ± 49| 758 ± 92  | 1771 ± 140|                                               |
| Ala132| 24 ± 4           | 16 ± 1 | 28 ± 1    | 241 ± 5   |                                               |
| Ala320| N.A.             | 27 ± 2 | 44 ± 7    | N.A.      |                                               |
| Ala656| N.A.             | N.A.   | N.A.      | 659 ± 18  |                                               |

[^a] Standard assay was performed as described in the text. Numbers are the average ± S.D. No cyclase activity was shown as N.A.

[^b] Each 2 μg of recombinant GST-fused proteins of CmCPS or CmKS was supplied into the standard assay mixture containing [^3H]GGDP and the site-directed mutant.

**Fig. 3. Cyclase activities of CT and NT mutant proteins.** Substrate [^3H]GGDP and GST-fused truncated proteins were used for cyclase assay. Calculated molecular sizes of these truncated proteins (except for GST moiety, 26 kDa) are shown in this figure. The FCPS/KS wild type (WT) is 946 amino acid polypeptides, and its molecular size is 106 kDa. Deduced amino acid sequence near the N-terminal region of the wild-type (1-409 amino acid residues, black area) shares homology with plant CPS and other diterpene cyclases. The residual region near the C terminus (537 amino acid residues, gray area) shows no homology with other terpene cyclases, including plant KS. White boxes show aspartate/glutamate-rich motifs. The broken regions show truncated domains.  

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The CPS gene transcript levels (39) but also GGDP levels. In contrast to CPS, the activity of plant diterpene cyclases like abi-
found in KS. Deletion of 108 amino acid residues from the C terminus of Arabidopsis KS eliminated enzyme activity, indicating that the C terminus of KS contains essential amino acid residues for this activity (8). Several residues in the C-terminal region of KS are conserved with essential amino acids involved in the active site of tobacco 5-epi- aristolochene synthase (8, 29). Because the CT-900 protein lacks KS activity, the C-terminal region contains essential amino acid residues for the formation of the active site. However, conserved amino acids in tobacco 5-epi-aristolochene synthase and KS are not found in FCPS/KS. The NT-44 protein lacking CPS activity showed lower KS activity than the NT-359 mutant (Fig. 3). In addition, site-directed mutagenesis of the Asp-132 residue of FCPS/KS affected both CPS and KS reactions (Table III). These results suggest that the N-terminal region of FCPS/KS may contain important motifs for both CPS reaction and binding of the substrate CDP for KS reaction. This speculation is supported from the facts that the N-terminal region near the SAYDTAW motif of CmCPS is necessary to maintain cyclase activity (6) and that this region shares high similarity among many diterpene cyclases cloned from plants (44–45). Recently, a cDNA for ent-kaurene synthase was cloned from G. fujikuroi (31), and its recombinant protein showed a bifunctional cyclase activity (46). Deduced amino acid sequences of CPS/KS between diterpene biosynthetic enzymes between fungi and plants.

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