Structural and Biochemical Elucidation of Mechanism for Decarboxylative Condensation of β-Keto Acid by Curcumin Synthase

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The typical reaction catalyzed by type III polyketide synthases (PKSs) is a decarboxylative condensation between acyl-CoA (starter substrate) and malonyl-CoA (extender substrate). In contrast, curcumin synthase 1 (CURS1), which catalyzes curcumin synthesis by condensing feruloyl-CoA with a diketide-CoA, uses a β-keto acid (which is derived from diketide-CoA) as an extender substrate. Here, we determined the crystal structure of CURS1 at 2.32 Å resolution. The overall structure of CURS1 was very similar to the reported structures of type III PKSs and exhibited the αβαβα fold. However, CURS1 had a unique hydrophobic cavity in the CoA-binding tunnel. Replacement of Gly-211 with Phe greatly reduced the enzyme activity. The crystal structure of the G211F mutant (at 2.5 Å resolution) revealed that the side chain of Phe-211 occupied the hydrophobic cavity. Biochemical studies demonstrated that CURS1 catalyzes the decarboxylative condensation of a β-keto acid using a mechanism identical to that for normal decarboxylative condensation of malonyl-CoA by typical type III PKSs. Furthermore, the extender substrate specificity of CURS1 suggested that hydrophobic interaction between CURS1 and a β-keto acid may be important for CURS1 to use an extender substrate lacking the CoA moiety. From these results and a modeling study on substrate binding, we concluded that the hydrophobic cavity is responsible for the hydrophobic interaction between CURS1 and a β-keto acid, and this hydrophobic interaction enables the β-keto acid moiety to access the catalytic center of CURS1 efficiently.

Type III polyketide synthases (PKSs) are distributed in diverse organisms, including plants, fungi, and bacteria, and are responsible for the syntheses of various biologically and pharmaceutically important compounds (1, 2). The universal reactions catalyzed by type III PKSs are as follows: (i) transfer of acyl-CoA (called the starter substrate) to the catalytic Cys, resulting in an acyl-PKS complex; (ii) decarboxylation of malonyl-CoA (called the extender substrate) to form an active anion; (iii) Claisen condensation of the active anion with the acyl moiety attached to the catalytic Cys to generate an acyl-CoA that has an additional two-carbon unit; (iv) extension of the polyketide chain by repeating reactions i–iii; and (v) cyclization of the resultant polyketide chain and release from the enzyme (1, 2). As an example, the reaction catalyzed by chalcone synthase (CHS), which is a type III PKS, is depicted in Fig. 1A (1, 2). The most important amino acid residues for type III PKSs are Cys-164, His-303, and Asn-336 (numbering in CHS), which create a catalytic triad. Cys-164 in CHS forms a thioester bond with p-coumaroyl-CoA (starter substrate) and a polyketide intermediate. His-303 and Asn-336 are responsible for acyl-PKS complex formation and the decarboxylative condensation of malonyl-CoA (1, 2). Type III PKSs form homodimers, and each monomer has a characteristic structure, called the αβαβα fold (3–7). The catalytic triad lies in the middle of each molecule and is connected to the surface by a CoA-binding tunnel, so-called because cocrystallization of CoA with several type III PKSs revealed that the CoA molecule bound to this tunnel (4–7). Thus, this tunnel probably enables an acyl moiety of acyl-CoA (or malonyl-CoA) to access the catalytic center efficiently (4, 5). On the opposite side of the CoA-binding tunnel, a cavity exists that accommodates the growing polyketide chain and facilitates cyclization of the polyketide chain (4–7). The diverse polyketides produced by type III PKSs arise from the specificity for starter and extender substrates, the number of incorporated extender substrates, and the cyclization pattern of the polyketide intermediate.

Until recently, the extender substrate used by type III PKSs was believed to be limited to malonyl-CoA and its derivative CoAs (e.g. methylmalonyl-CoA). However, we recently discovered a novel class of type III PKSs that uses a β-keto acid as an extender substrate; this class catalyzes the decarboxylative condensation between an acyl-CoA and a β-keto acid, resulting in unusual head-to-head condensation of polyketide chains (8–11). Curcumin synthases 1–3 (CURS1, CURS2, and CURS3) (9, 10) from Curcuma longa catalyze formation of curcumin by condensing feruloyl-CoA with the β-keto acid,
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FIGURE 1. Reactions catalyzed by chalcone synthase (A) and by curcumin synthase 1 (B and C). A, CHS catalyzes condensation of p-coumaroyl-CoA and three molecules of malonyl-CoA to synthesize the tetraketide intermediate. The resulting tetraketide intermediate is further cyclized by CHS and converted to naingenin chalcone. B, CURS1 catalyzes the hydrolysis of diketide-CoA to yield a β-keto acid (ii) and decarboxylative condensation of the β-keto acid with feruloyl-CoA to yield curcumin (iii). CURS1 barely catalyzes the formation of diketide-CoA from feruloyl-CoA and malonyl-CoA (i). In C. longa, this reaction is primarily catalyzed by a diketide-CoA synthase. C, for analysis of the activity of CURS1, cinnamoylferuloylmethane (curcuminoid) formation from feruloyl-CoA with cinnamoyldiketide-NAC (an analog of diketide-CoA) or 3-oxo-5-phenyl-4-pentenoic acid (a β-keto acid) was examined.

5-(4-hydroxy-3-methoxyphenyl)-3-oxo-4-pentenoic acid. Curcuminoid synthase from Oryza sativa catalyzes the in vitro formation of bisdemethoxycurcumin by condensing p-coumaroyl-CoA with the β-keto acid, 5-(4-hydroxyphenyl)-3-oxo-4-pentenoic acid. In addition to these enzymes, ginger, banana, and red root (Wachendorfia thyrsiflora) have been believed to possess some type III PKSs of this class (12–14). Furthermore, PqsD, a 3-oxoketoacyl-acyl carrier protein synthase III (KASIII) related to type III PKSs, is predicted to catalyze the condensation between anthraniloyl-CoA and a 3-oxo-4-pentenoic acid. In addition to these enzymes, ginger, banana, and red root (Wachendorfia thyrsiflora) have been believed to possess some type III PKSs of this class (12–14). Furthermore, PqsD, a 3-oxoketoacyl-acyl carrier protein synthase III (KASIII) related to type III PKSs, is predicted to catalyze the condensation between anthraniloyl-CoA and a 3-oxo-4-pentenoic acid. In PqsD, using a diketide-CoA as the extended substrate, CURS1 catalyzes the formation of diketide-CoA from feruloyl-CoA and malonyl-CoA (i). In C. longa, this reaction is primarily catalyzed by a diketide-CoA synthase. CURS1 is its ability to use a β-keto acid as an extended substrate. However, the only direct evidence that supports this mechanism is detection of condensation activity between feruloyl-CoA and a β-keto acid in vitro. Thus, further experiments are required to clarify how CURS1 catalyzes condensation between feruloyl-CoA and diketide-CoA. Two questions are raised regarding condensation between feruloyl-CoA and a β-keto acid. First, does CURS1 catalyze the decarboxylative condensation of a β-keto acid using the identical mechanism as that for the normal decarboxylative condensation of malonyl-CoA by typical type III PKSs? Second, how does CURS1 bind the extended substrate that lacks a CoA moiety? To address these questions, we determined the crystal structure of CURS1 at 2.32 Å resolution, followed by site-directed mutagenesis, biochemical experiments, and a modeling study. We succeeded in providing the first structural insights into the molecular mechanism for the unique reaction catalyzed by CURS1.

EXPERIMENTAL PROCEDURES

Materials—Escherichia coli strains JM109 and BLR(DE3), plasmid pUC19, restriction enzymes, T4 DNA ligase, Taq DNA polymerase, PrimeSTAR HS DNA polymerase, and other DNA-modifying enzymes were purchased from Takara.
Biochemicals (Shiga, Japan). Plasmids pET16b and pET26b were purchased from Novagen (Darmstadt, Germany). Ferulic acid and curcumin were purchased from Wako Chemicals (Osaka, Japan). Cinnamoylferuloylmethane, cinnamoyldiketide N-acetylcysteamine (NAC), dihydrocinnamoyldiketide-NAC, 3-oxooctanoyl-NAC, and 3-oxopalmityl-NAC were synthesized as described previously (8, 9). Plasmid pET16b-CURS1 was described previously (9), 3-Oxo-5-phenyl-4-pentenoic acid methyl ester was synthesized as described previously (8, 9). Acetoacetic acid and 3-oxoacrylic acid methyl ethers were purchased from Tokyo Chemical Industry (Tokyo, Japan). To avoid the nonenzymatic decarboxylation of 3-oxo-5-phenyl-4-pentenoic acid, acetoacetic acid, and 3-oxoacrylic acid, the acids were prepared by alkaline hydrolysis of 3-oxo-5-phenyl-4-pentenoic acid, acetoacetic acid, and 3-oxoacrylic acid methyl esters immediately before use.

Construction of pET26b-CURS1—Using pET16b-CURS1 (9) as a template, the DNA fragment encoding curcumin synthase was amplified by PCR with primer 5′-CCGAATTC-CATAAGGCACACCTTGACGTT-3′ (underlining indicates the Ndel site, boldface letters indicate the EcoRI site, and italic letters indicate the start codon of the CURS1 gene) and 5′-CCGGATCTCTAGTTTGTTTGTTGTTGTT-TCAGTCGCAACTATGGGA-3′ (underlining indicates the BamHI site, boldface letters indicate four codons for His residues fused to the C terminus of CURS1, and italic letters indicate an artificially generated stop codon). Amplified fragment was cloned between EcoRI and BamHI sites of pUC19, resulting in pUC19-CURS1-His4. pUC19-CURS1-His4 was digested with Ndel and BamHI, and the DNA fragment encoding CURS1 with four His was cloned between Ndel and BamHI sites of pET26b, resulting in pET26b-CURS1-His4. An expression plasmid (pET26b-CURS1 (G211F)-His4) for production of CURS1 (G211F) with four His residues at its C terminus was constructed by replacing the 954-bp Ndel-PstI fragment of pET26b-CURS1-His4 with that of pET16b-CURS1 (G211F) (see below).

Production, Purification, and Crystallization of CURS1-His4—CURS1 with four His residues at its C terminus (CURS1-His4) was produced and purified as described below. The E. coli BL21 (DE3) strain harboring pET26b-CURS1-His4 was inoculated into 10 ml of Luria Bertani (LB) medium containing 50 mg/liter kanamycin and incubated at 37 °C overnight. A portion (7 ml) of the preculture was inoculated into 3 liters of LB medium containing a few drops of antifoam SI (Wako Chemicals, Osaka, Japan). Cinnamoylferuloylmethane, cinnamoyldiketide-NAC, 3-oxooc...
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![Image of molecular structure]

A unit cell of the CURS1 crystal contained four molecules of CURS1, two dimers. All the monomers are highly similar to each other, with root mean square deviations of 0.4. The overall structure of the CURS1 dimer was very similar to the structures of some type III PKSs and exhibited the αββα fold (Fig. 2, A and B) (1, 2, 4). Met-137 of each monomer was located adjacent to the active site pocket of the other monomer and created a wall beside the active site pocket, as reported for other type III PKSs (Fig. 2A) (4, 6, 19). The catalytic triad (Cys-164, His-303, and Asn-336) was buried in the middle of each monomer and was connected to the surface with a CoA-binding tunnel. The two catalytic centers of a dimer were apparently inaccessible to each other because no tunnel connected them. Thus, two active sites can work independently of each other, similar to other type III PKSs.

RESULTS

Overall Structure of CURS1—We determined the crystal structure of recombinant CURS1-His$_4$ at 2.32 Å resolution. A unit cell of the CURS1 crystal contained four molecules of CURS1, two dimers. All the monomers are highly similar to each other, with root mean square deviations of 0.4. The overall structure of the CURS1 dimer was very similar to the structures of some type III PKSs and exhibited the αββα fold (Fig. 2, A and B) (1, 2, 4). Met-137 of each monomer was located adjacent to the active site pocket of the other monomer and created a wall beside the active site pocket, as reported for other type III PKSs (Fig. 2A) (4, 6, 19). The catalytic triad (Cys-164, His-303, and Asn-336) was buried in the middle of each monomer and was connected to the surface with a CoA-binding tunnel. The two catalytic centers of a dimer were apparently inaccessible to each other because no tunnel connected them. Thus, two active sites can work independently of each other, similar to other type III PKSs.
Characteristics of the Active Site of CURS1—Similar to other type III PKSs, CURS1 had an active site pocket composed of three parts as follows: (i) a CoA-binding tunnel that represents the entrance for the substrates; (ii) the catalytic triad; and (iii) a cavity that could accommodate an extended polyketide chain (1–5). In addition to the catalytic triad (Cys-164, His-303, and Asn-336), two well conserved Phe residues, Phe-215 and Phe-265, were retained by CURS1 (Fig. 2C). These two Phe residues are called the gatekeepers (1, 2). However, the orientation of the Phe-265 side chain was different from that of the corresponding Phe residues (Phe-265) of CHS (Fig. 3B) (4). This difference resulted in a slightly wider CoA-binding tunnel of CURS1 in comparison with CHS. More importantly, the Phe-265 side chain of CURS1 provided a hydrophobic cavity in the CoA-binding tunnel. The hydrophobic cavity was surrounded by the side chains of Phe-265, Phe-215, Phe-267, and Gly-211 (Fig. 3A). In contrast, a cavity that would accommodate an extended polyketide chain in CURS1 was much narrower than that of CHS because of the substitution of Ser-338 (in CHS) by Gln-338 and the change in orientation of the Phe-265 side chain (Fig. 3B). Unexpectedly, near the catalytic Cys of each monomer, electron density existed that was probably derived from malonic acid, which was included in the crystallization buffer (Fig. 3 and supplemental Fig. S1). Although the orientation of malonic acid was somewhat different from monomer to monomer (supplemental Fig. S1E), malonic acid formed hydrogen bonds with the catalytic residues His-303 and Asn-336 in every monomer. This observation suggested that malonic acid could mimic the β-keto acid moiety of the extender β-keto acid. Malonic acid in chain A was depicted in Fig. 3, because its electron density was most clear among four malonic acid molecules included in the crystal structure (one each monomer) and because position of the molecule appeared to be significant as a β-keto acid analog; the center carbon of malonic acid should be located sufficiently close to the catalytic Cys.

Role of the Catalytic Triad in Decarboxylative Condensation of a β-Keto Acid and Starter Substrate Loading—To investigate the functional role of His-303 in β-keto acid condensation by CURS1, active site mutant enzymes (H303Q and
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H303A) were prepared. The curcuminoid synthesis activity of CURS1 was examined using two different enzyme assays. One involved curcuminoid synthesis from feruloyl-CoA and cinnamoyldiketide-NAC (Fig. 1C, ii and iii). The other was that from feruloyl-CoA and 3-oxo-5-phenyl-4-pentenoic acid (Fig. 1C, iii). The former included two reactions as follows: hydrolysis of cinnamoyldiketide-NAC and condensation of the resultant β-keto acid with feruloyl-CoA. In contrast, the latter included only condensation of the β-keto acid with feruloyl-CoA, allowing us to examine the direct influence of these mutations on β-keto acid condensation. Notably, we used cinnamoyldiketide-NAC and 3-oxo-5-phenyl-4-pentenoic acid as analogs of feruloyldiketide-CoA and 5-(4-hydroxy-3-methoxyphenyl)-3-oxo-4-pentenoic acid, respectively, because of the ease of their chemical synthesis. When either of the mutant CURS1 enzymes (CURS1 H303Q and CURS1 H303A) was used, the yield of the curcuminoid product decreased markedly in both assays (Fig. 4). This result suggested that His-303 is important for β-keto acid condensation. Notably, the corresponding CHS mutants (CHS H303Q and CHS H303A) similarly showed a marked decrease in the decarboxylative condensation of malonyl-CoA (23, 24).

We then determined the kinetic parameters of recombinant CURS1 H303Q and wild-type CURS1 in curcuminoid formation from feruloyl-CoA and cinnamoyldiketide-NAC, as well as that from feruloyl-CoA and 3-oxo-5-phenyl-4-pentenoic acid (Table 1). The $k_{cat}$ of curcuminoid formation from 3-oxo-5-phenyl-4-pentenoic acid was much higher than that from cinnamoyldiketide-NAC. This result indicated that the hydrolysis step is rate-limiting in curcuminoid formation from feruloyl-CoA and diketide-CoA, excluding the possibility that condensation precedes hydrolysis in the reaction. In the H303Q mutant, curcuminoid synthesis activity from 3-oxo-5-phenyl-4-pentenoic acid decreased ~10-fold in comparison with the wild-type enzyme. Decreased curcuminoid synthesis activity resulted from a marked reduction of the $k_{cat}$ value. Introduction of the H303Q mutation into CHS similarly altered its enzymatic property (24), suggesting that the catalytic His residue has the same function between CURS1 and CHS in the decarboxylative condensation of the β-keto acid and malonyl-CoA, respectively.

In CHS, His-303 is involved not only in the decarboxylative condensation of malonyl-CoA but also in starter substrate loading (23, 24). To obtain further insight into the role of His-303, we examined the dependence of the enzyme activity on pH in curcuminoid synthesis from feruloyl-CoA and 3-oxo-5-phenyl-4-pentenoic acid using the H303Q mutant and the wild-type enzyme (supplemental Fig. S2). In the wild-type enzyme, the optimum pH was 7.0, and almost no reduction in the activity occurred between pH 6.0 and 7.0. In contrast, in the H303Q mutant, the optimum pH was 7.5, and the activity gradually decreased when the pH was lowered. This difference could be explained by the role of His-303 in the starter substrate loading, which was described in analysis of the reaction mechanism of CHS (23, 24). In CHS, His-303 acts as the general base that takes the proton from the thiol moiety of Cys-164, which lowers the $pK_a$ of the thiol moiety (23, 24). The deprotonated thiol residue of Cys-164 reacts with a starter substrate to facilitate starter substrate loading. The replacement of His-303 by Gln results in an increase in the $pK_a$ of Cys-164, which raises the optimum pH of the starter substrate loading and decreases the activity under lower pH conditions (23, 24). Thus, this result indicated that His-303 in CURS1 may function similarly to the His-303 residue of CHS, not only in decarboxylative condensation of a β-keto acid but also in starter substrate loading.

Thus, all of these results supported the idea that CURS1 catalyzes decarboxylative condensation of a β-keto acid using the same mechanism as that for normal decarboxylative condensation of malonyl-CoA by typical type III PKSs. This idea was also supported by the following observations. First, the recombinant CURS1 protein demonstrated weak decarboxylation activity toward 3-oxo-5-phenyl-4-pentenoic acid (supplemental Fig. S3), similar to typical type III PKSs that have decarboxylation activity toward malonyl-CoA (23, 24).

**FIGURE 4.** Curcuminoid synthesis activity of the wild-type and mutant CURS1 enzymes from feruloyl-CoA with cinnamoyldiketide-NAC (gray bars) or 3-oxo-5-phenyl-4-pentenoic acid (β-keto acid) (white bars).

**TABLE 1**

| Kinetic parameters of recombinant CURS1 proteins in curcuminoid synthesis from feruloyl-CoA and cinnamoyldiketide-NAC or 3-oxo-5-phenyl-4-pentenoic acid (n = 3) |
|---------------------------------------------------------------|
| **Cinnamoyldiketide-NAC** | **3-Oxo-5-phenyl-4-pentenoic acid** |
| **WT** | **G211F** | **H303Q** | **WT** | **G211F** | **H303Q** |
| $k_{cat}$ (1/min) | 0.26 ± 0.01 | 0.088 ± 0.010 | 0.0044 ± 0.0002 | 7.5 ± 2.7 | 0.50 ± 0.09 | 0.10 ± 0.01 |
| $K_m$ (μM) | 1.7 ± 0.5 | 113 ± 6 | 3.0 ± 0.0 | 5.9 ± 1.7 | 138 ± 88 | 1.2 ± 0.3 |
| $k_{cat}/K_m$ (1/s/M) | 2915 ± 650 | 13 ± 1 | 24 ± 1 | 20541 ± 3487 | 112 ± 44 | 1605 ± 456 |

* $K_m$ values for feruloyl-CoA are shown.
ond, generation of a covalent bond between CURS1 and feruloyl-CoA was observed (supplemental Fig. S4). Third, the C164S mutation abolished the activity to synthesize curcuminoid from feruloyl-CoA and cinnamoyldiketide-NAC or a \(\beta\)-keto acid (data not shown). These results showed that the covalent bond between Cys-164 and the feruloyl moiety was necessary for \(\beta\)-keto acid condensation.

**Extender Substrate Specificity of CURS1**—To gain insight into the unique ability of CURS1 to use a \(\beta\)-keto acid as an extender substrate, we analyzed the extender substrate specificity of CURS1. CURS1 was incubated with several diketide-NAC (or -CoA) molecules or \(\beta\)-keto acids in the presence of feruloyl-CoA as a starter substrate. CURS1 used dihydrocinnamoyldiketide-NAC, 3-oxooctanoyl-NAC, and 3-oxopalmityl-NAC as extender substrates and produced curcuminoid derivatives (see supplemental Fig. S5 for their structures), suggesting that CURS1 has a relatively relaxed extender substrate specificity toward diketide-CoAs with a hydrophobic moiety. However, when CURS1 was incubated with acetoacetyl-CoA, acetoacetic acid, or 3-oxovaleric acid, no curcuminoid derivative was detected. This suggested that diketide-CoAs (\(\beta\)-keto acids) derived from short chain fatty acids were not used by CURS1. We assumed that hydrophobic interaction between CURS1 and a \(\beta\)-keto acid would be important, and thus a hydrophobic moiety (a phenyl group or a long alkyl chain) of a \(\beta\)-keto acid would be essential for the \(\beta\)-keto acid to be used as an extender substrate of CURS1.

**Involvement of the Hydrophobic Cavity around Phe-265 in \(\beta\)-Keto Acid Condensation**—All structurally characterized type III PKSs have a CoA-binding tunnel that serves as the entrance for the starter and extender substrates, and several amino acid residues in the tunnel are responsible for binding of the CoA moiety of the substrates (1, 2). Because CURS1 can use an extender substrate that lacks the CoA moiety, it may have an alternative mechanism for binding the extender substrate. As described above, we assumed that the hydrophobic interaction between CURS1 and a \(\beta\)-keto acid would be important. The hydrophobic cavity around Phe-265 in the CoA-binding tunnel was very likely to be involved in the hydrophobic interaction. Thus, we constructed two mutant CURS1 proteins, in which Gly-211 was replaced by the bulkier amino acids, Phe and Trp. Because Gly-211 is located in the hydrophobic cavity, an aromatic group of the side chain of the Phe-211 and Trp-211 of each of the mutant enzymes was assumed to occupy this hydrophobic cavity.

As expected, both CURS1 mutants (G211F and G211W) showed only weak activity in synthesizing curcuminoid from feruloyl-CoA and cinnamoyldiketide-NAC (or 3-oxo-5-phenyl-4-pentenoic acid) (Fig. 4). Subsequently, the steady-state kinetics parameters of the G211F mutant were determined (Table 1). In the G211F mutant, the \(K_m\) values for feruloyl-CoA were increased, and the \(k_{cat}\) values were reduced markedly in both reactions. However, this change might have been caused by the change in the structure of the catalytic triad. To eliminate this possibility, we determined the crystal structure of CURS1 G211F at 2.5 Å resolution (Fig. 5). Except for the phenyl group generated by the mutation, the structure of the active site pocket of CURS1 G211F was almost identical to that of wild-type CURS1 and only slightly affected the orientation of Phe-215 (Fig. 5). As expected, the phenyl group generated by the G211F mutation occupied the hydrophobic cavity around Phe-265 (Fig. 5). From these results, we concluded that occupation of the cavity by the side chain of Phe-211 generated by the G211F mutation resulted in a drastic decrease in the curcuminoid synthesis activity of CURS1. This result supported the idea that the hydrophobic cavity is responsible for the hydrophobic interaction between CURS1 and a \(\beta\)-keto acid, which is apparently essential for CURS1 to use an extender substrate lacking a CoA moiety. Thus, it was very likely that the reduced \(\beta\)-keto acid binding of the G211F mutant resulted in the drastic decrease in the \(k_{cat}\) values. However, we had no definite explanation for the increased \(K_m\) values of the G211F mutant for feruloyl-CoA. Conceivably, the G211F mutation inhibited feruloyl-CoA binding by restricting the flexibility of Phe-265. The flexibility of Phe-265 was predicted to be important for starter substrate binding as described below.

**Computational Modeling of CURS1 That Binds a Feruloyl Moiety and a \(\beta\)-Keto Acid**—To discuss the reaction catalyzed by CURS1, we used the molecular operating environment program and constructed a model of CURS1 that contained a feruloyl moiety covalently attached to Cys-164 and bound 5-(4-hydroxy-3-methoxy-phenyl)-3-oxo-4-pentenoic acid (a \(\beta\)-keto acid) in the active site pocket. The cavity that probably accommodates the starter substrate was found in the CURS1 structure, but it was somewhat small to accommodate the feruloyl moiety. This observation suggested that a conformational change of CURS1 could occur during starter substrate binding. Some type III PKSs have been predicted to require such a conformational change during their reaction, and Phe-265 (in CHS) is predicted to be responsible for this change (4,
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The orientation of Phe-258 (which corresponds with Phe-265 in CHS) is also known to be different between chain A and chain B in the crystal structure of BAS (19). These results suggested that flexibility of Phe-265 might be the common feature of type III PKSs. Thus, Phe-265 of CURS1 was assumed to move slightly to accommodate a feruloyl moiety and chain B in the crystal structure of BAS (19). These results were predicted by the Autodock program (25). We built several models in each of which β-keto acid was differently located. However, we could evaluate the significance of each model on the basis of the results of our biochemical analyses, which indicated that CURS1 catalyzes decarboxylative condensation of β-keto acid using the same mechanism as that for normal decarboxylative condensation of malonyl-CoA by typical type III PKSs. Finally, we selected a model as the most reliable one (Fig. 6B). The fact that the β-keto acid moiety in this model is located at a position similar to the malonic acid in the crystal structure of CURS1 chain A (supplemental Fig. S6) also supported this model.

In this model, the oxygen atom of the β-keto moiety was located sufficiently close to the catalytic His-303 and Asn-336 residues to interact with them (Fig. 6B). The α-carbon was also located close to the ketone carbon of the feruloyl moiety, so that the active anion formed by the decarboxylation of the β-keto acid could efficiently generate a carbon-carbon bond to the feruloyl moiety by nucleophilic attack (Fig. 6B). Thus, this model mimics the state immediately before decarboxylative condensation. Importantly, in this model, the phenyl group of the β-keto acid is located in the hydrophobic cavity around Phe-265 and is surrounded by the side chains of Phe-215, Phe-265, and Phe-267 (Fig. 6B). As described above, the phenyl group of Phe-211 generated by the G211F mutation occupied the cavity in the CURS1 G211F mutant that showed greatly reduced curcuminoid synthesis activity. This result supported the present model in which the phenyl group of the β-keto acid interacts with the hydrophobic cavity.

DISCUSSION

In this study, we determined the crystal structure of CURS1 followed by site-directed mutagenesis to examine the enzymatic properties and a modeling study on substrate binding.

From these results, we conclude that CURS1 catalyzes head-to-head condensation of polyketide chains using the following reactions (see Fig. 7). First, CURS1 catalyzes the transfer of the feruloyl moiety of feruloyl-CoA to the catalytic Cys-164. Diketide-CoA then enters the CoA-binding tunnel and is hydrolyzed by an unknown mechanism. The resulting β-keto acid subsequently accesses the catalytic triad and is used as an extender substrate. The hydrophobic cavity around Phe-265 in the CoA-binding tunnel is responsible for the binding of the β-keto acid; the phenyl moiety of the β-keto acid interacts with the hydrophobic cavity. Finally, decarboxylative condensation of the β-keto acid with the feruloyl moiety results in curcumin formation. CURS1 catalyzes the decarboxylative condensation of the β-keto acid using a mechanism identical to that for normal decarboxylative condensation of malonyl-CoA in typical type III PKSs.

We had assumed two possible mechanisms in which CURS1 could use a β-keto acid as an extender substrate. First, a hydrogen bond network between a β-keto acid and CURS1, which does not exist in other type III PKSs, might allow the β-keto acid to be close to the active site without help from the interaction between CoA and the CoA-binding tunnel. Second, an additional hydrophobic pocket around the catalytic triad might accommodate the phenyl group of the β-keto acid, so that the β-keto acid moiety could effectively gain access to the catalytic center. The malonic acid that was crystallized in the active site pocket of the wild-type CURS1 protein helped us to search for such a hydrogen bond network to stabilize the β-keto moiety. However, we could not find any hydrogen bonds to interact with the malonic acid, except those between the malonic acid and the catalytic His-303 and Asn-336 residues. Absence of a strong hydrogen bond network may explain the diversity of the orientation of malonic acid in the catalytic center of each CURS1 monomer. The observation that CURS1 could not use β-keto acids having a short alkyl chain as an extender substrate also indicated that such a hydrogen bond network should not exist between the β-keto acid moiety and CURS1. In contrast, we found a unique hydrophobic cavity in the CoA-binding tunnel and showed that this cavity was responsible for the binding of the

FIGURE 6. Model for the binding of a feruloyl moiety and a β-keto acid in the active site pocket of CURS1. A, native structure of the active site pocket of CURS1. B, most reliable model structure. A feruloyl moiety and a β-keto acid are colored magenta and green, respectively.
We conclude that CURS1 obtained its ability to use an extender substrate that lacks the CoA moiety by providing a hydrophobic cavity that accommodates a hydrophobic moiety (a phenyl group or a long alkyl chain) of a β-keto acid. In addition to the ability to use an extender substrate that lacks a CoA moiety, CURS1 possesses two characteristic features. First, CURS1 catalyzes the hydrolysis of diketide-CoAs. From the kinetics analysis of the two curcuminoid synthesis reactions (one from feruloyl-CoA and cinnamoyldiketide-NAC and the other from feruloyl-CoA and 3-oxo-5-phenyl-4-pentenoic acid), the hydrolysis of diketide-CoA was revealed to be the rate-limiting reaction in the curcuminoid synthesis from feruloyl-CoA and the diketide-CoA. In BAS, diketide-CoA is predicted to be loaded on the catalytic Cys just as a starter substrate and then hydrolyzed by nucleophilic attack of a water molecule that was activated by the catalytic His (19). However, we assumed that the feruloyl moiety derived from the starter substrate (feruloyl-CoA) would be bound to the catalytic Cys of CURS1 when hydrolysis of a diketide-CoA (extender substrate) occurs (Fig. 7). Thus, CURS1 probably uses a different catalytic mechanism to hydrolyze diketide-CoA. Second, CURS1 exhibited very weak activity for the normal head-to-tail condensation of feruloyl-CoA with malonyl-CoA. Asn-306 in CURS1 may be responsible for its low efficiency in the condensation of malonyl-CoA. The corresponding amino acid residue to Asn-306 is Gly or Ser in other type III PKSs. Asn-306 is located at the wall of the CoA-binding tunnel (Fig. 3A) and may inhibit the malonyl moiety of malonyl-CoA in approaching the catalytic center. In contrast, a cavity that accommodates the feruloyl moiety is located on the opposite side to the CoA-binding tunnel in the active site pocket. Thus, Asn-306 may not affect starter substrate loading. Further experiments, including cocrystallization of CURS1 with substrates and site-directed mutagenesis, are necessary to determine the mechanisms of these two features of CURS1.

Although the crystal structure of PqsD was previously reported, the study was primarily focused on dihydroxyquinoline synthesis, which does not require β-keto acid condensation, and its activity to condense a β-keto acid has not been confirmed to date (15). Thus, this study is the first report to predict the mechanism of condensation of a β-keto acid, an extender substrate that lacks the CoA moiety, by a ketosynthase. Because the head-to-head condensations of polyketide chains by ketosynthases exist not only in plants but also in bacteria and they seem to be preceded by the reaction similar to the CURS1 reaction, this study is of significance for understanding the biochemistry of these natural products.

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