Structural basis for the formation of acylalkylpyrones from two β-ketoacyl units by the fungal type III polyketide synthase CsyB

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Running title: Structure of the acylalkylpyrone synthase CsyB

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Background: CsyB from Aspergillus oryzae catalyze the condensation of two β-ketoacyl units.

Result: Crystal structures of CsyB revealed a novel pocket for accommodating the acetoacetyl-CoA starter.

Conclusion: CsyB catalyzes the remarkable one-pot condensation of two β-ketoacyl units within a single active-site.

Significance: Structure-function analyses of CsyB provide insights into molecular bases for polyketide coupling reactions.

ABSTRACT

The acylalkylpyrone synthase CsyB from Aspergillus oryzae catalyzes the one-pot formation of the 3-acyl-4-hydroxy-6-alkyl-α-pyrones (AcAP) scaffold, from acetoacetyl-CoA, fatty acyl-CoA, and malonyl-CoA. This is the first type III polyketide synthase (PKS) that performs not only the polyketide chain elongation but also the condensation of two β-ketoacyl units. The crystal structures of wild-type CsyB and its I375F and I375W mutants were solved at 1.7 Å, 2.3 Å and 2.0 Å resolutions, respectively. The crystal structures revealed a unique active-site architecture featuring a hitherto unidentified novel pocket for accommodation of the acetoacetyl-CoA starter, in addition to the conventional elongation/cyclization pocket with the Cys-His-Asn catalytic triad and the long hydrophobic tunnel for binding the fatty acyl chain. The structures also indicated the presence of a putative nucleophilic water molecule, activated by the hydrogen bond networks with His377 and Cys155 at the active-site center. Furthermore, in vitro enzyme reaction confirmed that the 18O atom of the H218O molecule is enzymatically incorporated into the final product. These observations suggested that the enzyme reaction is initiated by the loading of acetoacetyl-CoA onto Cys155, and subsequent thioester bond cleavage by the nucleophilic water generates the β-keto acid intermediate, which is placed within the novel pocket. The second β-ketoacyl unit is then produced by polyketide chain elongation of fatty acyl-CoA with one molecule of malonyl-CoA, and the condensation with the β-keto acid generates the final products. Indeed, steric modulation of the novel pocket by the structure-based I375F and I375W mutations resulted in altered specificities for the chain lengths of the substrates.
The structurally simple type III polyketide synthases (PKSs) utilize a single active-site to catalyze iterative condensations of CoA thioesters and cyclization of the poly-β-keto intermediates, to generate various natural product scaffolds with remarkable biological activities (1-3). The acylalkylpyrone synthase CsB from Aspergillus oryzae is the first type III PKS that catalyzes not only the polyketide chain elongation but also the one-pot condensation of two β-ketoacetyl units, to produce the 3-acyl-4-hydroxy-6-alkyl-α-pyrene (AcAP) scaffold (Fig. 1A) (4-7). Thus, CsB accepts acetoacetyl-CoA, fatty acyl-CoA, and malonyl-CoA as substrates, and performs the decarboxylative condensation of malonyl-CoA with fatty acyl-CoA to produce the β-ketoacetyl diketide, which then reacts with acetoacetyl-CoA to yield 3-acyl-4-hydroxy-6-alkyl-α-pyrene (2), a putative precursor of csypryone B (Fig. 1A). In addition, the in vitro enzyme reaction also yields dehydroacetic acid (3-acyl-4-hydroxy-6-methyl-α-pyrene) (1) and 3-acyl-4-hydroxy-6-alkyl-α-pyrene (3), by the coupling of two molecules of acetoacetyl-CoA and the β-ketoacetyl diketide intermediate, respectively.

The A. oryzae CsB shares 26% amino acid sequence identity with the Mycobacterium tuberculosis alkylpyrone synthase PKS18 (8) and 37% identity with the Neurospora crassa 2′-oxoalkylresorcylic acid synthase (ORAS) (9), which produce tri-/tetraketide alkylpyrones and pentaketide alkylresorcylic acids by iterative condensations of fatty acyl-CoA with two/three molecules and four molecules of malonyl-CoA, respectively (Figs. 1B, 1C, and 2). Previous crystallographic studies of M. tuberculosis PKS18 (8) and N. crassa ORAS (9) revealed a long hydrophobic tunnel for binding the fatty acyl chain. This tunnel extends from the active-site to the protein surface, and is primarily generated by subtle changes of the backbone dihedral angles in the core of the protein. Thus, the functional diversity of the type III PKS enzymes is considered to be derived from slight modifications of the active-site architecture (8-18).

To clarify the structural details of the remarkable one-pot formation of the AcAP scaffold, and to further understand the structure-function relationships of the type III PKS enzymes, we now report the crystal structures of the wild-type and mutants of A. oryzae CsB. The crystal structures revealed the unique active-site architecture of CsB, featuring a previously unidentified novel pocket for the accommodation of the acetoacetyl-CoA starter, in addition to the conventional elongation/cyclization pocket with the Cys-His-Asn catalytic triad (10), and the long hydrophobic fatty acyl binding tunnel, as observed in M. tuberculosis PKS18 (8) and N. crassa ORAS (9). Furthermore, the presence of a putative nucleophilic water molecule, activated by the hydrogen bond networks at the active-site center, suggested an unusual mechanism for the CsB-catalyzed one-pot formation of the AcAP scaffold.

**EXPERIMENTAL PROCEDURES**

**Materials** – Fatty acyl-CoA, acetoacetyl-CoA, malonyl-CoA, and H$_2^{18}$O were obtained from Sigma-Aldrich. Acyl-diketide-NACs with various lengths were synthesized according to the published method (19, 20). Oligonucleotides were obtained from Eurofins Genomics.

**Compound analysis** – Online LC-ESIMS spectra were measured with an Agilent Technologies HPLC 1100 series, coupled to a Bruker Daltonics esquire4000 ion trap mass spectrometer fitted with an ESI source. HRESIMS spectra were measured with an Agilent 1100 series HPLC–microTOF mass spectrometer (JEOL), using electrospray ionization.

**Compounds informations** – 3-butyryl-4-hydroxy-6-propyl-2H-pyran-2-one (3a). LC-ESIMS (positive): Rt = 19.8 min. MS, m/z 225 [M+H]$^+$, 207, 167, 155. UV: $\lambda_{max}$ 311 nm. HRMS (ESI): found for [C$_{12}$H$_9$O$_4$]$^+$ 223.10052; calcd. 223.09703.

6-butyryl-4-hydroxy-3-pentanoyl-2H-pyran-2-one (3b). LC-ESIMS (positive): Rt = 22.9 min. MS, m/z 253 [M+H]$^+$, 235, 181, 169. UV: $\lambda_{max}$ 312 nm. HRMS (ESI): found for [C$_{14}$H$_{15}$O$_4$]$^+$ 251.12979; calcd. 251.12833.

3-hexanoyl-4-hydroxy-6-pentyl-2H-pyran-2-one (3c). LC-ESIMS (positive): Rt = 24.9 min. MS, m/z 281 [M+H]$^+$, 263, 195, 183. UV: $\lambda_{max}$ 312 nm. HRMS (ESI): found for [C$_{16}$H$_{23}$O$_4$]$^+$ 279.16399; calcd. 279.15963.

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3-heptanoyl-6-hexyl-4-hydroxy-2H-pyran-2-one (3d). LC-ESIMS (positive): Rt = 27.0 min. MS, m/z 309 [M+H]^+). 291, 209, 197. UV: λmax 312 nm. HRMS (ESI): found for [C_{13}H_{25}O_4]^+ 307.19303; calcd. 307.19093.

6-heptyl-4-hydroxy-3-octanoyl-2H-pyran-2-one (3e). LC-ESIMS (positive): Rt = 28.5 min. MS, m/z 337 [M+H]^+. 319, 223, 211. UV: λmax 312 nm. HRMS (ESI): found for [C_{20}H_{34}O_4]^+ 335.26273; calcd. 335.26223.

4-hydroxy-3-nonanoyl-6-octyl-2H-pyran-2-one (3f). LC-ESIMS (positive): Rt = 32.1 min. MS, m/z 365 [M+H]^+. 347, 237, 225. UV: λmax 312 nm. HRMS (ESI): found for [C_{22}H_{35}O_4]^+ 363.25843; calcd. 363.25353.

Structure determination – Expression, purification, and crystallization of the recombinant A. oryzae CsyB were performed as previously reported (21). The initial phases of the CsyB structure were determined by molecular replacement, using the N. crassa ORAS structure (PDB ID 3EUT) as the search model. Molecular replacement was performed with MOLREP, in the CCP4 suite (22, 23). The structure was modified manually with Coot (24) and refined with PHENIX (25). The final crystal data and intensity statistics are summarized in Table 1. A structural similarity search was performed, using the Dali program (26). The cavity volumes were calculated with the program CASTP (http://cast.engr.uic.edu/cast/).

All crystallographic figures were prepared with PyMOL (DeLano Scientific, http://www.pymol.org). The refined model includes residues 14-389 in chains A and B, respectively, and 695 molecules of water.

Site-directed mutagenesis, expression, and purification – The plasmids expressing the mutants of A. oryzae CsyB (C123A, S; A265V, F; I375F, W; and H377P, F) were constructed with a QuikChange Site-Directed Mutagenesis Kit (Stratagene), according to the manufacturer’s protocol by using the following pairs of primers (mutated codons are underlined): C123A (5'-CATTTGGGCTGTAACGGGACGAATAC AG-3') and 5'-GGAACGCTTATCAGCCGTTACAGC C-3'), C123S (5'-GATTACGCTGTAACGGGACGAATAC AG-3') and 5'-GGAACGCTTATCAGCCGTTACAGC C-3'), A265V (5'-CAAAGGGTATCGTGTTATGACAAAC -3') and 5'-GATTACGCTGTAACGGGACGAATAC AG-3'), I375F (5'-GATGCCATGGCCAAAAGCTGACAGGACC -3'), I375W (5'-GATGCCATGGCCAAAAGCTGACAGGACC -3'), and 5'-GATGCCATGGCCAAAAGCTGACAGGACC-3'), H377P (5'-GGCAGCTATCGGCCCGCGCATACGCTGG -3') and 5'-GGCAGCTATCGGCCCGCGCATACGCTGG -3'), H377F (5'-GGCAGCTATCGGCCCGCGCATACGCTGG -3') and 5'-GGCAGCTATCGGCCCGCGCATACGCTGG -3'). The mutant proteins were expressed and purified according to the same procedure used for the wild-type CsyB, and were concentrated to 10 mg/ml in 20 mM Tris-HCl (pH 7.5) buffer, containing 200 mM NaCl and 2 mM DTT.

Enzyme assay of CsyB and mutants – The enzyme assay and the product identification were performed as previously reported (6), except for the use of an elution program with a linear gradient of CH_3CN from 20% to 100% in H_2O containing 0.05% TFA for 30 min, which was maintained at 100% for 20 min further, to separate the reaction products. The H_3O^+ labeling experiment was performed under the same conditions for the wild-type enzyme, except for the replacement of H_2O with H_2^{18}O.

Kinetic parameters of CsyB – A standard reaction contained various chain length of diketide-NAC (concentration varied between 0.5 and 1.25 mM) and the purified enzyme in 50 mM potassium...
phosphate buffer, pH 8.0, in a total volume of 100 µl. After preincubation at 37 °C for 5 min, the reactions were initiated by adding the purified enzyme and then further incubated at 37 °C for 20 min before quenching with 20 µl of 6 M HCl and 80 µl of methanol. The decrease of substrate were quantified by the HPLC analysis using a COSMOSIL C18-MS-II column (4.6 × 250 mm, Nacalai Tesque) eluted isocratically with 40% acetonitrile containing 0.05% TFA at a flow rate of 1.0 ml/min. Steady-state kinetic parameters were determined from Lineweaver-Burk plots.

Crystallization and structure determination of the I375F and I375W mutants – The I375F mutant crystals were grown at 20°C, with a 5 mg/ml protein solution in 100 mM Pipes-NaOH (pH 6.5) buffer, containing 6% (w/v) PEG 4,000 and 250 mM lithium chloride, by using the sitting-drop vapor-diffusion method. The I375W mutant crystals, complexed with CoA-SH, were obtained in 100 mM Pipes-NaOH (pH 6.5) buffer, containing 6% (w/v) PEG 4,000, 250 mM lithium chloride, and 2 mM CoA-SH, with a 4.5 mg/ml protein solution. Both crystals were independently transferred into the reservoir solution with 18% (v/v) glycerol as a cryoprotectant, and then flash cooled at 100 K in a nitrogen-gas stream. Both X-ray diffraction data sets were collected at NW12 of Photon Factory-AR (wavelength, 1.0000 Å), by using an ADSC Quantum 210r CCD detector. The final model of CsyB I375F consists of residues 13-389 of chain A, 13-364 and 366-389 of chain B, and 393 molecules of water. On the other hand, the final model of CsyB I375W consists of residues 14-361 and 366-389 of chain A, 13-389 of chain B, and 655 molecules of water. Crystal data and intensity statistics are summarized in Table 1. Both crystals belonged to space group P21, and the unit cell dimensions were very similar to those of wild-type CsyB. The initial phases of the CsyB mutant structures were determined by molecular replacement, using the wild-type CsyB structure as the search model. Molecular replacement and refinement were performed in the same manner as for the wild-type enzyme. Refinement statistics are given in Table 1. The coordinates and structure factors have been deposited in the Protein Data Bank, www.pdb.org (PDB ID 3WXY, 3WXZ, and 3WY0 for the A. oryzae CsyB CoA-SH complexed structure, CsyB I375F mutant, and CsyB I375W CoA-SH complexed structure, respectively).

Molecular Modeling – The three-dimensional models of the enzyme-bound intermediates were generated by the Chem3D Ultra 13 program (CambridgeSoft). The intermediate models were manually swapped with the catalytic Cys155 in the CsyB structure by using Coot, and the energy minimization calculation by simulated annealing with PHENIX was then performed. The parameters of the intermediates for the energy minimization calculation were obtained by the PRODRG server (http://davapc1.bioch.dundee.ac.uk/prodrg/).

RESULTS

Overall structure of A. oryzae CsyB – The crystal structure of the wild-type CsyB, as a complex structure with CoA-SH, was solved by the molecular replacement method and refined to 1.7 Å resolution (Table 1). The asymmetric unit contained two nearly identical monomers, with root-mean-square deviations (RMSDs) of 0.1 Å for the Ca-atoms. The overall structure of the homodimeric CsyB revealed the conservation of the αβαβα thiolase fold, as observed in other type III PKSs (Fig. 3A) (1-3). The catalytic triad of Cys155, His310, and Asn343 is buried deep within each monomer and sits at the intersection of a 16 Å-long CoA binding tunnel and a large internal cavity, in a location and orientation very similar to those of the previously reported plant and microbial type III PKSs (8-18). The CoA-binding tunnel is connected to the protein surface, thus facilitating the loading of the substrate into the catalytic center (Fig. 3B). The overall structure of CsyB is thus highly homologous to those of other type III PKSs, including N. crassa ORAS (9), Gerbera hybrida 2-pyrene synthase (2-PS) (11), and Oryza sativa CUS (15), with RMSDs of 1.8 Å, 1.9 Å, and 2.0 Å, respectively, for the Ca-atoms.

In A. oryzae CsyB, the conserved active-site "gatekeeper" Phe265 (in CHS numbering) is characteristically substituted with Ala265 (Figs. 2 and 4A) (1, 2). A similar large-to-small substitution is also observed in the previously reported curcuminoïd-producing O. sativa CUS, which catalyzes the coupling reaction of a β-
ketoacyl diketide intermediate with coumaroyl-CoA (15). As in the case of CUS, we propose that this substitution not only expands the active-site entrance but also facilitates the movement of the enzyme-bound β-ketoacyl intermediate. Indeed, when Ala265 was replaced with Phe or Val, both mutants almost completely lost the enzyme activity (Fig. 5). On the other hand, the active-site Ser338 (in CHS numbering), in the neighbor of the catalytic Cys155, is substituted with a hydrophobic Ile345 (Figs. 2 and 4A). The hydrophobic substitution is also observed in several type III PKSs, including the alklypyrone-producing M. tuberculosis PKS18 (8) and methylpyrone-producing G. hybrida 2-PS, which is thought to be important for modulation of the catalytic activity (11). Most importantly, Pro375 (in CHS numbering), conserved in all type III PKSs, is uniquely substituted with His377, which plays an important role in the construction of the active-site architecture, as discussed later (Fig. 4D-F). Moreover, another residue, His128, corresponding to Met137 of CHS, protrudes into the other monomer by the formation of a cistepptide bond between His128 and Pro129 on a loop. However, in contrast to many of the typical type III PKSs, His128 is not involved in the formation of part of the active-site wall, as observed in M. tuberculosis PKS18 (8), N. crassa ORAS (9), and O. sativa CUS (15). Finally, CsyB has the second Cys123 in the active-site; however, when it was mutated to Ser or Ala, no significant change in the AcAP-producing activity was observed, indicating that the residue is not essential for the enzyme activity.

**Active-site architecture of A. oryzae CsyB** – The crystal structures revealed that the active-site of A. oryzae CsyB is composed of three sections (Fig. 4A): (i) the conventional elongation/cyclization pocket with the Cys-His-Asn catalytic triad, which is connected to the substrate entrance through the 16 Å-long CoA-binding tunnel (10), (ii) the downward expanding hydrophobic tunnel for binding the medium-length (C$_2$-C$_{15}$) fatty acyl chain, which extends from the active-site center to the protein surface, as also observed in the alklypyrone-producing M. tuberculosis PKS18 (8) and the alkylresorcylic acid-producing N. crassa ORAS (9), and (iii) the CsyB-specific novel pocket for the accommodation of the acetoacetyl-CoA starter or the short-chain (C$_2$-C$_2$) β-ketoacyl diketide unit, as discussed below.

Notably, most of the amino acid residues constituting the elongation/cyclization pocket are superimposable in nearly identical positions in the plant and microbial type III PKS enzymes (Fig. 4A). On the other hand, in the elongation/cyclization pocket, Ile254 (in CHS numbering) is characteristically altered to Ala254 in CsyB. As a result of this large-to-small substitution, the size of the elongation/cyclization pocket of CsyB (581 Å$^3$) is 1.2 times larger than those of M. sativa CHS (485 Å$^3$) and N. crassa ORAS (489 Å$^3$).

The fatty acyl chain-binding tunnel of CsyB (ca. 12 Å long) is significantly shorter than those of the longer (C$_{12}$-C$_{20}$) fatty acyl chain-accepting M. tuberculosis PKS18 (17 Å) and N. crassa ORAS (20 Å) (Fig. 4A-C). These tunnels are formed by subtle conformational changes in the regions corresponding to residues 204–209 in PKS18 and 185–190 in ORAS, as compared to the region of residues 193–198 in the plant CHS, respectively. In CsyB, this conformational change is also observed in the corresponding region of residues 189–193; however, the presence of the bulky side chains of the CsyB-specific Leu334 and Trp74 make the tunnel shorter than those of PKS18 and ORAS (Fig. 4A-C). As a result, the estimated total cavity volume of CsyB (1,056 Å$^3$) is smaller than those of M. tuberculosis PKS18 (1,682 Å$^3$) and N. crassa ORAS (1,495 Å$^3$), but it is large enough to accommodate the fatty acyl chain (C$_2$-C$_{15}$) of the substrate and the diketide intermediate, which is incorporated into the 6-alkyl moiety of the AcAP scaffold.

The CsyB-specific novel additional pocket, adjacent to the conventional elongation/cyclization pocket, is about 8 Å long, and consists of hydrophobic residues including Ala254, Ile271, Leu274, Ile375, and Leu381 (Figs. 4D and 6A). The backbone torsion angle of Ile375 (-86, 126) is slightly shifted by a φ angle of -43° and a ψ angle of +14°, in comparison with that of Phe373 (-129, 140), and the side chain of Ile375 protrudes toward Thr293. In contrast, the Phe373 residue in CHS, and the corresponding residues in other type III PKSs, protrude in a totally different direction, toward Phe371 in CHS (Fig. 4D-F).
Putative nucleophilic water molecule at the catalytic center – In the previously reported crystal structures of the plant type III PKSs, such as the stilbene-producing *Pinus sylvestris* STS (12), the curcuminoïd-producing *O. sativa* CUS (15), and the benzalacetone-producing *Rheum palmatum* BAS (16), thioesterase-like electronic hydrogen bond networks and activated water molecules (Fig. 4G-J) play critical roles in the enzyme reactions. For example, *P. sylvestris* STS utilizes the nucleophilic water molecule, activated by the so-called "aldol-switch" hydrogen bond network neighboring the catalytic Cys (Fig. 4H), for thioester bond cleavage of the enzyme-bound intermediate to produce the stilbene scaffold (12). On the other hand, in *O. sativa* CUS (15) and *R. palmatum* BAS (16), the cleavage of the thioester bond of the enzyme-bound intermediate by the activated water molecules (Fig. 4I and 4J) terminates the polyketide chain elongation at the diketide stage, to generate the β-keto diketide acid intermediates. The crystal structure of *A. oryzae* CsyB revealed the presence of a similar putative nucleophilic water molecule, which forms hydrogen bond networks with the CsyB-specific His377 and the main chain of the catalytic Cys155 at the active-site center (Figs. 3B and 4G). When His377 was changed to Pro or Phe, both mutants completely lost the enzyme activity (Fig. 5). These observations suggested that His377 plays important roles in the activation of a water molecule and the thioester bond cleavage of the enzyme-bound intermediate. However, only the presence of the putative nucleophilic water molecule at the active-site center does not exclude the possibility that the CsyB-catalyzed enzyme reaction proceeds without the involvement of the water molecule. For example, the coupling of the enzyme-bound β-ketoacyl intermediate with the "β-ketoacyl-CoA", but not with the "β-keto acid", would afford the AcAP scaffold (Fig. 7). In this case, the oxygen atom from the water molecule is not incorporated into the final product. To test this possibility, we performed the enzyme reaction in the presence of H$_2^{18}$O, using the C$_5$-diketide-NAC as a substrate. As a result, the obtained product 3b afforded parent ion peaks [M + H]$^+$ at m/z 253 and m/z 255, in addition to m/z 251, indicating the incorporation of two $^{18}$O atoms into the product (Fig. 8A). The percentage yields of m/z 251, m/z 253, and m/z 255 were 24%, 45%, and 31%, respectively (Table 2). Furthermore, when the $^{18}$O-labeled 3b was incubated in H$_2^{18}$O, the m/z 255 peak was dramatically decreased, and when unlabeled 3b was incubated in H$_2$O, the m/z 253 peak newly appeared after 5 hours (Fig. 8B).

Steric modulation of the CsyB-specific novel additional pocket – To further understand the intimate structural details of the CsyB-catalyzed enzyme reaction, we performed site-directed mutagenesis of the CsyB-specific Ile375, which is the crucial residue for the creation of the novel pocket for the acetoacyl-CoA starter binding. As a result, the I375W mutant lost the enzyme activity, whereas the I375F mutant maintained comparable activity to the wild-type CsyB (Fig. 5).

The diffraction-quality protein crystals of the I375F and I375W mutants were obtained in similar conditions to the wild-type CsyB, and the apo-crystal structure of I375F mutant and the crystal structure of I375W mutant with CoA-SH were solved at 2.3 Å and 2.0 Å resolution, respectively. The overall structures of I375F and I375W mutants were nearly identical to that of the wild-type, with RMSDs of 0.2 Å and 0.4 Å, respectively. Interestingly, the hydrogen bond network-activated water molecule is also conserved in the structures of both mutants. The total active-site cavity volume of the I375F mutant was increased from 1,056 Å$^3$ to 1,135 Å$^3$. This is due to the loss of the Cγ-methyl group of isoleucine and the subtle molecular movement of the Cα-atoms of Cys278 toward the opposite side of the novel pocket, by the replacement of the C8-methyl group of isoleucine with the bulkier aromatic ring (Fig. 6B). In contrast, the cavity size of the I375W mutant was decreased to 983 Å$^3$. The backbone torsion angle of Trp375 (∠-72, 134), in comparison with that of Ile375 (∠-86, 126), is slightly shifted by a φ angle of -14° and a ψ angle of -8°, and the side chain of Trp375 inclines toward the novel pocket. Furthermore, Ile253 and Ala254 protrude toward the novel pocket generated by the large movement of residues 247-255 in the loop region. In addition to the small-to-large substitution, the conformational change and the movement of the
loop region decreased the size of the active-site pocket of the I375W mutant (Fig. 6C).

The expansion of the CsyB-specific novel additional pocket by the I375F substitution suggested that the substrate and product specificities of the enzyme reaction may be altered. To test this hypothesis, we performed the enzyme reactions using various lengths of \( \beta \)-ketoacetyl diketide-NAC thioesters as substrates. The wild-type CsyB accepts two molecules of a short-chain (C\(_4\)-C\(_7\)) \( \beta \)-ketoacetyl diketide-NAC, and catalyzes coupling reactions to yield the single products \( 3b - 3d \), with various lengths of the alkyl and acyl chains (Figs. 6D). Interestingly, the I375F mutant, with the expanded pocket, accepted all of the (C\(_4\)-C\(_9\)) \( \beta \)-ketoacetyl diketide-NAC substrates to yield the unnatural novel products \( 3b - 3f \) (Figs. 6D). The steady-state kinetics values of CsyB wild-type and I375F mutant for C\(_4\)-C\(_6\) \( \beta \)-ketoacetyl diketide-NAC substrates were summarized in Table 3. The kinetic parameters for the reactions of C\(_4\)-C\(_9\) \( \beta \)-ketoacetyl diketide-NAC substrates were not determined because of too low efficacy of these reactions. The steady-state kinetics values of CsyB I375F for \( \beta \)-ketoacetyl diketide-NAC were ~1.2 folds better than those of CsyB wild-type.

**DISCUSSION**

In this study, we solved the crystal structures of the novel type III PKS CsyB from *A. oryzae* and its mutants. The crystal structures revealed a unique active-site architecture featuring a hitherto unidentified novel pocket, in addition to the conventional elongation/cyclization pocket with the Cys-His-Asn catalytic triad and the 12 Å length of hydrophobic tunnel for binding the fatty acyl chain. Furthermore, the hydrogen bond networks among a water molecule, the CsyB-specific His377, and the main chain of the catalytic Cys155, were observed at the active-site center. The site-directed mutagenesis of His377 suggested that this residue is crucial for the enzyme activity, presumably in the activation of a water molecule and the thioester bond cleavage of the enzyme-bound intermediate.

On the basis of these findings, we propose that the CsyB-catalyzed one-pot formation of the AcAP scaffold is initiated by the loading of acetoacetyl-CoA (or a short-chain \( \beta \)-ketoacyl diketide unit) onto the catalytic Cys155 at the active-site. The subsequent reorientation of the enzyme-bound \( \beta \)-ketoacyl unit (Fig. 9A), and the thioester bond cleavage by the nucleophilic water molecule, activated by the hydrogen bond networks with His377 and Cys155, generate the "first" \( \beta \)-ketoacyl unit, which is placed within the novel additional pocket (Fig. 9B). The fatty acyl-CoA (or the second acetoacetyl-CoA) is then loaded onto the free catalytic Cys155, and decarboxylative condensation with malonyl-CoA yields the enzyme-bound "second" \( \beta \)-ketoacyl diketide unit (Fig. 9C and 9D). The polyketide chain elongation of the fatty acyl substrate is terminated at this diketide stage, by a nucleophilic attack from the activated methylene of the "first" \( \beta \)-ketoacyl diketide unit and the thioester bond cleavage (Fig. 9E). The subsequent formation of the lactone generates the final AcAP products (Fig. 9F and 9G).

Notably, the *in vitro* enzyme reaction also yields dehydroacetic acid (3-acetyl-4-hydroxy-6-methyl-\( \alpha \)-pyrone) (1) and 3-acetyl-4-hydroxy-6-alkyl-\( \alpha \)-pyrone (3), by the coupling of two molecules of acetoacetyl-CoA and the short-chain \( \beta \)-ketoacyl diketide intermediate, respectively. Two crucial points of the CsyB-catalyzed enzyme reaction are the termination of the fatty acyl chain elongation at the diketide stage and the generation of the "second" \( \beta \)-ketoacyl diketide unit for the final coupling reaction. Notably, triketide or tetraketide pyrone by-products are not formed by the condensations of fatty acyl-CoA with two or three molecules of malonyl-CoA.

Interestingly, the *in vitro* H\(_2^{18}\)O-incorporation experiment indicated that one of the two \( ^{18}\)O atoms is enzymatically incorporated in to the product, most likely by the nucleophilic cleavage of the thioester bond of the Cys-bound intermediate, to generate the "\( \beta \)-keto acid" as the intermediate (while the other \( ^{18}\)O atom is exchangeable and spontaneously incorporated into the molecule). These results thus excluded the possibility of the "\( \beta \)-ketoacyl-CoA" intermediate, but suggested that CsyB catalyzes the coupling of the "\( \beta \)-keto acid" intermediate and the enzyme-bound \( \beta \)-ketoacyl unit (Fig. 7A). This is the first direct evidence that a type III PKS utilizes a water molecule to produce the \( \beta \)-keto acid intermediate during the enzyme reaction.
The small novel pocket, created by the CsyB-specific residues, including His377 and Ile375, is about 8 Å and has enough space to accept short-chain acyl-diketide acids (Figs. 4D and 6A). Indeed, wild-type CsyB accepts up to C7-diketide-NAC as the substrate, producing α-pyrone with varying chain length at the C-3 position. Furthermore, the in vitro analyses of I375F and I375W mutants, along with their crystal structures, revealed that the elimination of the pocket causes loss of the coupling activity, while its expansion results in broader substrate specificity for the extender substrates (Fig. 6D). These results indicated that CsyB utilizes this novel pocket for accommodation of the acetoacetoyl-CoA starter (or a short-chain β-ketoacyl diketide unit), and the size of pocket determines the chain length of the β-ketoacyl diketide which is incorporated into the 3-acyl moiety of the AcAP scaffold.

To the best of our knowledge, O. sativa CUS is the only type III PKS that catalyzes the one-pot condensation of a β-ketoacyl diketide intermediate with an enzyme-bound acyl substrate (Fig. 1D), but this is not the coupling of "two" β-ketoacyl units. In this case, CUS accepts two molecules of 4-coumaroyl-CoA and one molecule of malonyl-CoA as substrates. The previous crystallographic studies revealed that CUS utilizes a similar activated water molecule to generate a 4-coumaroyldiketide acid intermediate, which is then kept within the active-site, and the subsequent "decarboxylative" condensation with the second coumaroyl unit yields the linear curcuminoid scaffold (15).

In contrast, CsyB catalyzes the coupling of "two" β-ketoacyl units, in a "non-decarboxylative" manner. Similar coupling reactions were reported for the non-homologous bacterial ketosynthases (KSs), involved in the biosynthesis of 2,5-dialkylpyrones and 2,5-dialkylresorcinols (27-30). For example, CorB and MxnB, from the myxobacteria Coralloccocus coralloides and Myxococcus fulvus, catalyze the coupling of two β-ketoacyl diketide intermediates to produce the α-pyrene antibiotics corallopyronin and myxopyronin, respectively (27, 28). Furthermore, in photopyrone biosynthesis in Photorhabdus luminescens, PpyS produces a signaling pyrone molecule in a similar manner (29). On the other hand, the bacterial ketosynthese DarB catalyzes the coupling of a β-ketoacyl diketide intermediate and an α,β-unsaturated acyl precursor, thereby generating the dialkylcyclohexanedione scaffold, which is further converted to 2,5-dialkylresorcinols by the aromatase DarA in Chitinophaga pinensis (30).

Although the fungal CsyB does not share any sequence similarities with these bacterial KSs, the model structure of DarB predicted the presence of a CsyB-like additional pocket in the active-site (30). This suggests that these non-homologous bacterial KSs also utilize similar active-site architectures to catalyze their coupling reactions.

In conclusion, the crystallographic studies of the fungal A. oryzae CsyB provided the structural basis for the remarkable one-pot formation of the AcAP scaffold, by the condensation of two β-ketoacyl units. These findings provide further strategies toward expanding the catalytic repertoire of the versatile type III PKS enzymes and producing structurally divergent, biologically active novel polyketides for drug discovery.

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**FOOTNOTES**

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* T.M., H.M. and I.A. designed the experiments. T.M., D.Y., T.M. performed the experiments. T.M., H.M., I.F., and I.A. analyzed the data. T.M., H.M., and I.A. wrote the paper.

1The abbreviations used are: PKS, polyketide synthase; AcAP, 3-acyl-4-hydroxy-6-alkyl-α-pyrone; OARS, 2'-oxoalkylresorcylic acid synthase; CHS, chalcone synthase; BAS, benzalacetone synthase; CUS, curcuminoid synthase; 2-PS, 2-pyrole synthase; STS, stilbene synthase.
FIGURE LEGENDS

FIGURE 1. Reaction schemes of the synthesis of polyketides. (A) acylalkylpyrones from fatty acyl-CoA, malonyl-CoA, and acetoacetyl-CoA by CsyB (B) triketide alkyl pyrones from C_{12}-C_{20} fatty acyl-CoA and two molecules of malonyl-CoA by PKS18, (C) pentaketide alkylresorcylic acid from C_{4}-C_{20} fatty acyl-CoA and four molecules of malonyl-CoA by ORAS, (D) bisdemethoxycurcumin from two molecules of 4-coumaroyl-CoA and one molecule of malonyl-CoA by CUS.

FIGURE 2. Comparison of the primary sequences of A. oryzae CsyB and other type III PKSs. AoCsyB, A. oryzae PCS; AoCsyA, A. oryzae CsyA; NcORAS, N. crassa ORAS; OsCUS, O. sativa CUS; MtPKS18, M. tuberculosis PKS18. The secondary structures of CsyB are also delineated: α helices (blue rectangles), β strands (orange arrows), and loops (red, bold lines) are diagrammed. The catalytic triad of Cys-His-Asn is highlighted red. The residues thought to be important for the steric modulation of the active-site in a number of divergent type III PKSs are highlighted in blue.

FIGURE 3. Overall structures of A. oryzae CsyB and close-up view of its active-site. (A) Overall structure of A. oryzae CsyB wild-type. The structure is represented by cartoon model. The catalytic Cys155 and the substrate entrance are represented by a CPK model and an arrow, respectively. The CoASH molecules is magenta stick model. (B) The Fo-Fc density map of the active-site residues, CoASH, and putative nucleophilic water molecule in monomer A, countered at +1.0 sigma. The density of CoA and water molecules are represented as dark green mesh. The water molecule and the hydrogen bonds are indicated with red sphere and green dotted lines, respectively. Black, red, and blue arrows indicate the orientation of CoA binding tunnel, novel pocket, and fatty acyl chain-binding tunnel, respectively.

FIGURE 4. Comparison of the active-site structures of A. oryzae CsyB and other type III PKSs. (A)-(C) Comparison of the active-site cavity of (A) A. oryzae CsyB, (B) N. crassa ORAS, and (C) M. tuberculosis PKS18. The C_{16}-fatty acid molecule in N. crassa ORAS is shown as a green stick model. (D)-(F) Close-up views of the novel pocket of (D) A. oryzae CsyB and corresponding regions of (E) N. crassa ORAS, (F) M. tuberculosis PKS18. The arrows indicate the entrance of cavity. The elongation/cyclization pocket, the downward expanding hydrophobic tunnel, and the CsyB-specific novel pocket, described in result, are colored pink, green, and blue, respectively. The residues mutated in this study are labeled red color. (G)-(J) Close-up views of the electronic hydrogen bond networks of (G) A. oryzae CsyB, (H) P. sylvestris STS, (I) O. sativa CUS, and (J) R. palmatum BAS. Catalytic residues are highlighted by red square. The water molecule and the hydrogen bonds are indicated with red spheres and green dotted lines, respectively.

FIGURE 5. The results of mutagenesis analyses. HPLC elution profiles of enzyme reaction products of the wild-type CsyB and its I375F, I375W, A265F, A265V, H377F, and H377P mutant enzymes.

FIGURE 6. Close-up views of the novel pockets of CsyB and its mutants. (A) CsyB wild-type, (B) CsyB I375F, and (C) CsyB I375W. (D) The substrate specificities of wild-type CsyB and the I375F mutant for various lengths of diketide-SNAC substrates.

FIGURE 7. A scheme of acylalkylpyrone forming from (A) β-keto acid and (B) β-ketoacyl CoA. The β-keto acid is produced by cleavage of enzyme-bound intermediate using activated water molecule. Red highlighted atoms are derived from water molecule.

FIGURE 8. Comparisons of HR-ESI-MS spectra. (A) 3b produced by CsyB in 80% of H_{2}{^{18}}O, and the products of further incubation with {^{18}}O-labeled 3b in H_{2}{^{16}}O without enzyme and (B) 3b produced by CsyB in H_{2}{^{16}}O, and the products of further incubation with non-labeled 3b in H_{2}{^{18}}O without enzyme. (C) The structure of 3b and m/z of {^{18}}O incorporated 3b.
FIGURE 9. Proposed mechanism for the CsyB enzymatic reaction. (A–F) Three-dimensional model of (A) reorientation of the diketide intermediate covalently bound to the catalytic Cys155, (B) hydrolysis of the "first" β-ketoacyl diketide unit, (C) loading of the fatty acyl-CoA onto the catalytic Cys155, (D) proton abstraction from the stored β-ketoacyl diketide unit, (E) coupling reaction between two β-ketoacyl units, and (F) lactamization to produce the final products. (G) Schematic representation of the proposed mechanism.
## Table 1. Data collection, phasing, and refinement statistics.

| Unit cell parameter                  | CsyB WT         | CsyB I375F       | CsyB I375W       |
|--------------------------------------|-----------------|------------------|------------------|
| Space group                          | $P2_1$          | $P2_1$           | $P2_1$           |
| $a, b, c$ (Å)                        | 70.0, 104.8, 73.5 | 69.9, 104.8, 73.2 | 70.1, 104.6, 73.7 |
| $\beta$ ($^\circ$)                  | 114.4           | 114.3            | 114.4            |
| Resolution range (Å)                 | 50.00-1.71 (1.81-1.71) | 50.00-2.30 (2.44-2.30) | 50.00-2.00 (2.12-2.00) |
| Completeness (%)                     | 96.3 (95.3)     | 99.5 (99.1)      | 99.6 (99.1)      |
| <$I/\sigma I>$ (%)                   | 17.3 (5.3)      | 22.3 (5.5)       | 13.6 (3.8)       |
| $R_{merge}$ † (%)                   | 5.2 (26.3)      | 5.3 (26.8)       | 7.7 (35.5)       |
| Redundancy                           | 3.8 (3.9)       | 3.8 (3.7)        | 3.8 (3.7)        |
| No. of observed reflection           | 388,694 (62,153) | 161,189 (25,112) | 247,674 (38,739) |
| No. of unique reflection             | 101,115 (16,084) | 42,426 (6,766)   | 65,058 (10,375)  |

**Refinement**

| Resolution (Å)                      | 39.5-1.71       | 41.2-2.30        | 41.2-2.00        |
| Overall $R_{work}$ (%)             | 17.5            | 17.3             | 17.3             |
| Overall $R_{free}$ (%)             | 19.9            | 22.3             | 20.4             |
| Total atoms                         | 6523            | 6139             | 6476             |
| No. of protein atoms                | 5730            | 5746             | 5723             |
| No. of waters                       | 695             | 393              | 655              |
| No. of ligand                       | 96              | -                | 96               |
| Average B-factors (Å²)              | 22.8            | 32.3             | 17.5             |
| protein atoms                       | 32.9            | 33.9             | 26.3             |
| waters                               | 34.0            | -                | 32.1             |
| ligands                              |                 |                  |                  |
| r. m. s. d. from ideal              |                 |                  |                  |
| bond length (Å)                     | 0.007           | 0.007            | 0.007            |
| bond angles (°)                     | 1.150           | 1.038            | 1.068            |

† $R_{merge} = \Sigma_{hkil} \Sigma_i I_i(hkl) - \langle I(hkl) \rangle / \Sigma_{hkil} \Sigma_i I_i(hkl)$, where $I(hkl)$ is the intensity of reflection $hkl$, $\Sigma_{hkil}$ is the sum over all reflections, and $\Sigma_i$ is the sum over $i$ measurements of reflection $hkl$. 
TABLE 2. Extent of $^{18}$O incorporation in 3b under different conditions.

| m/z   | Enzyme reaction in H$_2^{18}$O | Enzyme reaction in H$_2^{16}$O | Incubation of non-labeled 3b in H$_2^{18}$O | Incubation of $^{18}$O-labeled 3b in H$_2^{16}$O |
|-------|-------------------------------|-------------------------------|----------------------------------|----------------------------------|
| 251 (0 $^{18}$O) | 100                          | 24                           | 30                              | 45                              |
| 253 (1 $^{18}$O) | 0                            | 45                           | 70                              | 52                              |
| 255 (2 $^{18}$O) | 0                            | 31                           | 0                               | 3                               |
TABLE 3. Steady-state kinetic parameters of CsyB wild-type and I375F mutant enzymes.

| Substrate          | $K_M$ (mM) | $k_{cat}$ (min$^{-1}$) | $k_{cat}/K_M$ (s$^{-1}$M$^{-1}$) |
|--------------------|------------|------------------------|----------------------------------|
| C$_4$-diketide NAC | 6.4 ± 1.2  | 27.5 ± 5.8             | 72.3                             |
| C$_5$-diketide NAC | 3.1 ± 1.6  | 17.0 ± 6.6             | 90.2                             |
| C$_6$-diketide NAC | 6.8 ± 1.6  | 42.7 ± 8.1             | 104.9                            |

| Substrate          | $K_M$ (mM) | $k_{cat}$ (min$^{-1}$) | $k_{cat}/K_M$ (s$^{-1}$M$^{-1}$) |
|--------------------|------------|------------------------|----------------------------------|
| C$_4$-diketide NAC | 7.1 ± 2.6  | 37.2 ± 11.1            | 87.5                             |
| C$_5$-diketide NAC | 1.4 ± 0.6  | 8.7 ± 2.5              | 92.9                             |
| C$_6$-diketide NAC | 4.5 ± 1.6  | 32.9 ± 5.4             | 122.2                            |
FIGURE 1

A. 1x CoAS \( \rightarrow \) CoA \( \rightarrow \) CoA \( \rightarrow \) 3-acetyl-4-hydroxy-6-alkyl-\( \alpha \)-pyrone (\( R = n-C_11 \), others: 2), \( R = n-C_11 \cdot C_{17} \)

B. 2x CoA \( \rightarrow \) CoA \( \rightarrow \) CoA \( \rightarrow \) C_{11} \cdot C_{19} alkyl pyrone (\( R = n-C_{11} \cdot C_{19} \))

C. 4x CoA \( \rightarrow \) CoA \( \rightarrow \) CoA \( \rightarrow \) C_{4} \cdot C_{20} \cdot 2'-oxoalkylresorcylic acid (\( R = n-C_{3} \cdot C_{19} \))

D. 1x CoA \( \rightarrow \) CoA \( \rightarrow \) CoA \( \rightarrow \) bisdemethoxycurcumin
FIGURE 2

Structure of the acylalkylpyrone synthase CsyB
FIGURE 3

A

B

Structure of the acylalkylpyrone synthase CsyB
Structure of the acylalkylpyrone synthase CsyB
Structure of the acylalkylpyrone synthase CsyB

FIGURE 5

Wild-type
I375F
I375W
A265F
A265V
H377F
H377P

5.0 10.0 15.0 20.0 25.0
Structure of the acylalkylpyrone synthase CsyB

FIGURE 7

A

B

EnzS

EnzS

SCoA
Structure of the acylalkylpyrone synthase CsyB

FIGURE 8

A

Enzyme reaction in H₂¹⁶O

Further incubation in H₂¹⁶O for 5 hrs

B

Enzyme reaction in H₂¹⁶O

Further Incubation in H₂¹⁸O for 5 hrs

C

0¹⁸O : m/z 251
1¹⁸O : m/z 253
2¹⁸O : m/z 255
FIGURE 9

Structure of the acylalkylpyrone synthase CsyB