博士論文 (要約)

Structural basis of two novel ketosynthases that catalyze the non-decarboxylative Claisen condensation
（脱炭酸を伴わないクライゼン縮合を触媒する新規ケトシンターゼに関する構造解析）

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Introduction

Ketosynthases (KSs) are essential for fatty acid, polyketide, and mevalonate biosynthesis. They include both KS domain of large multi-domain enzymes like type I fatty acid synthase (FAS) and type I polyketide synthase (PKS) and stand-alone enzymes as type II FAS, type II PKS, type III PKS, thiolase II. The natural function of all KSs is responsible for formation of carbon and carbon bond *via* Claisen condensation reaction by addition of two-carbon units. In Claisen condensation reaction, there are two types of reactions: non-decarboxylative and decarboxylative condensation depending on how to generate the nucleophilic α-anion. Two types of FASs and three types of PKSs all perform the decarboxylative condensation. So, this kind of reaction has been extensively studied. While only thiolase II, OleA, MxnB and CorB catalyze the non-decarboxylative condensation. Until now, examples of non-decarboxylative ketosynthases are still scarce.

CsyB, a unique KS from fungi *Aspergillus oryzae*, catalyzes the consecutive condensation of fatty acyl-CoA (**1**) with a dearboxylative unit malonyl-CoA (**2**) and a non-dearboxylative unit acetoacetyl-CoA (**3**), to produce a new pyrone scaffold, 3-acetyl-4-hydroxy-6-alkyl-α-pyrone (**4**) (Fig. 1A).

In contrast, StlD, a novel KS, performs the non-decarboxylative condensation of a β-ketoacyl thioester isovaleryl β-ketoacyl-SNAC (**5**) and a α, β-unsaturated acyl thioester 5-phenyl-2, 4-pentadienoyl-SNAC (**6**) to form the isopropylstilbene along with StlC, a corresponding aromatase (Fig. 1B).

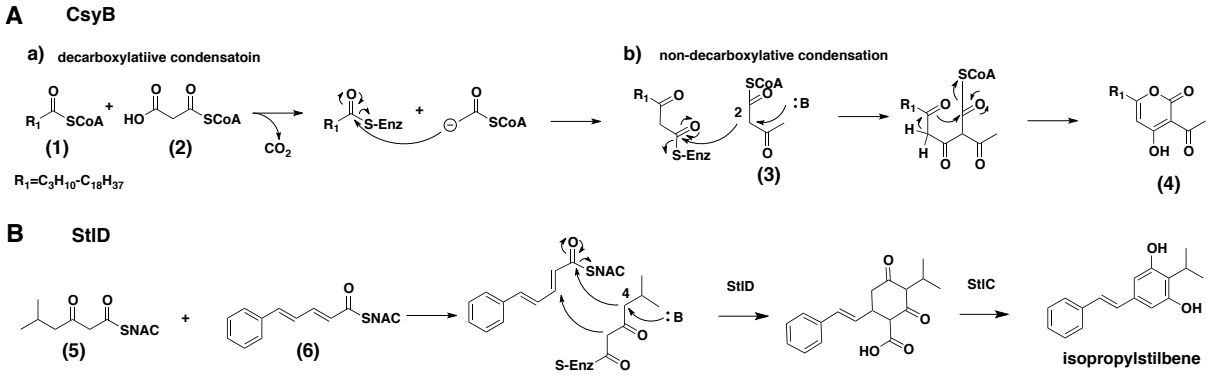

Fig.1 Catalytic reaction of two novel ketosynthases CsyB and StlD

Therefore, these two enzymes both catalyze the uncommon non-decarboxylative condensation.

In addition, they are both different from the known non-decarboxylative KSs.

1) Catalytic unit: They use an unusual unit β-ketoacyl thioester as the substrate instead of acetyl-CoA.

2) Activation mode: Their catalytic triad Cys-His-Asn, conserved in decarboxylative reaction, is different from non-decarboxylative catalytic triad Cys-His-Cys as thiolase, which utilize the second Cys to initiate the reaction. In addition, these two enzymes lack the E117 as OleA to activate the substrate.

Furthermore, there is difference between CsyB and StlD in their reaction mechanism.

1) CsyB is the first example that catalyzes both decarboxylative and non-decarboxylative condensation;

whereas StlD performs the reaction that includes in non-decarboxylative condensation and Michael addition.

2) CsyB initiates the reaction from the C_2 position of compound **(3)**; while StlD enable activate the less acidic C_4 position of compound **(5)** (Fig. 1).

To clarify the catalytic mechanism of these two enzymes, in this study, the major targets are to 1) understand the structural basis of CsyB and StlD; 2) elucidate the catalytic mechanism of CsyB and StlD; 3) enzymatically synthesize novel compounds using rationally engineered enzymes and unnatural substrates.

Results and discussion

1.1 Overall structure and active site architecture of CsyB

The crystal structure of wild-type (WT) CsyB, as a complex structure with CoA-SH, was solved by molecular replacement method and refined to 1.7 Å resolution. The overall structure of the homodimeric CsyB revealed the conservation of the αβαβα thiolase fold. The catalytic triad of C155, H310 and N343 is buried deeply within each monomer.

Based on the crystal structure of CsyB, several putatively functional amino acid residues were found: 1) H377, which is proposed to be responsible for initiating of the enzymatic reaction. 2) I375, which is maybe involved in controlling the volume of unidentified novel cavity. 3) An unidentified novel cavity, which is composed of A254, I271, L274, I375 and L381 (Fig. 2B). It is proposed that this cavity accepts the long acyl group of alkyl β-ketoacyl-SNAC because it specifically exists in CsyB compared with other type III PKSs, *Neruospora crassa* ORAS and *Oryza sativa* CUS.

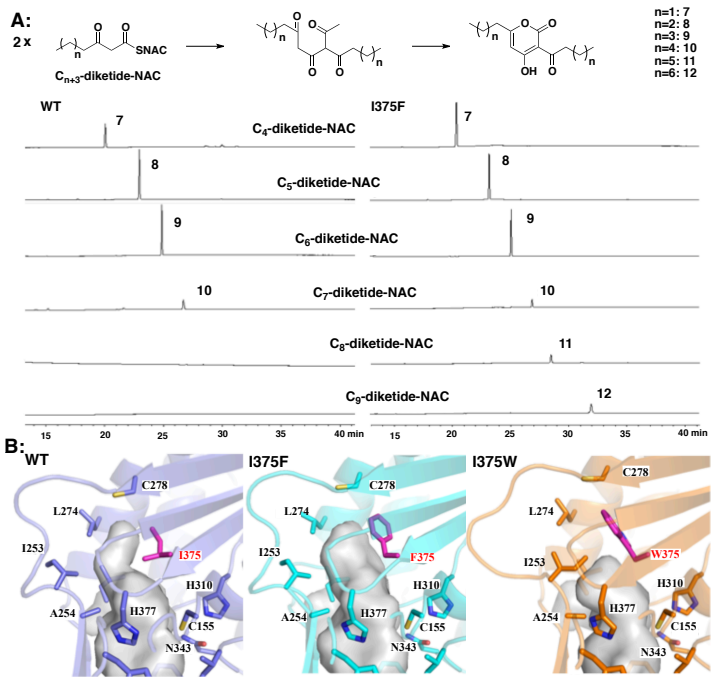

Fig.2 A: The substate specificities of WT CsyB and I375F; B: Close-up views of novel pockets of CsyB and its mutants

1.2 Enzyme assay of CsyB and its mutants using unnatural substrate

To test whether I375 substitution alters the substrate specificity, I performed the enzyme reactions using various lengths of β-ketoacyl-SNAC thioesters as substrates. The WT CsyB accepts two molecules of short-chain C_4~C_7 β-ketoacyl-SNAC, and catalyzes coupling reactions to yield the products **7-10**. The activities of CsyB mutants I375F and I375W were measured. Surprisingly, CsyB I375F, which introduces a bulky aromatic group in side chain, accepts all of β-ketoacyl-SNAC substrates from C_4 to C_9 to yield the unnatural novel products **7-12** (Fig. 2A). This result suggests that the CsyB specific novel cavity is indeed

responsible for accepting the acyl group of alkyl β-ketoacyl-SNAC and its volume is directly controlled by I375 amino acid residue.

1.3 Crystal structures of CsyB mutants I375F and I375W

To further understand the function of I375, crystal structures of two mutants I375F and I375W were solved at 2.3 and 2.0 Å, respectively. The structures provide direct evidences that the size of the novel cavity in mutant I375F is enlarged compared with WT enzyme; while in I375W, the bulky substitution occupied almost the entire cavity resulting in complete loss of activity (Fig. 2B).

1.4 $H_2{}^{18}O$ labeling experiment

To demonstrate whether water molecule, which is in the presence of the active site center, is involved in the CsyB catalytic process, stable isotope $H_2{}^{18}O$ labeling assay was performed. Based on the result of LC-MS, it is confirmed that ^{18}O atom is incorporated into the final product. Therefore, the catalytic mechanism should be involved in water molecule and hydrogen bond network.

1.5 Proposed catalytic mechanism

The catalytic mechanism is proposed based on the crystal structures, mutational experiment and feeding the stable isotope assay. The reaction is initiated by the loading of a β-ketoacyl thioester unit onto the catalytic C155 at the active site. The subsequent reorientation of the enzyme-bound β-ketoacyl unit happens by moving into the novel additional pocket. Then the nucleophilic water molecule, activated by the hydrogen bond network with H377 and C155, cleaves thioester bond. The "first" β-ketoacyl unit is released. Fatty acyl-CoA is then loaded onto the free catalytic C155, and decarboxylative condensation with malonyl-CoA yields the "second" β-ketoacyl unit. 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" β-ketoacyl unit. At the same time, the thioester bond is cleavaged. The subsequent cyclization of the lactone generates the final products (Fig. 3).

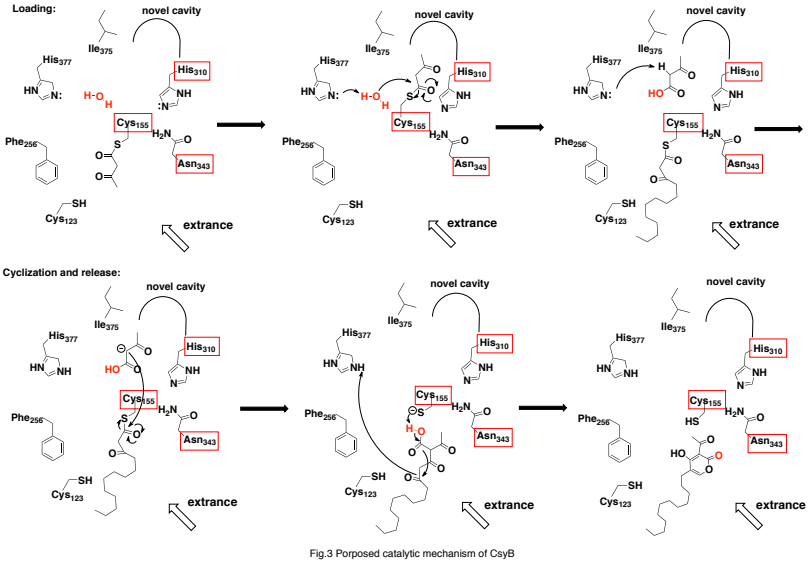

Fig.3 Porposed catalytic mechanism of CsyB

2.1 Overall structure and active site architecture a of StlD

The crystal structure of WT StlD was solved by single-wavelength anomalous diffraction method and refined to 2.3 Å resolution. The conserved αβαβα thiolase fold is observed in structure of StlD. Its conserved catalytic

triad consists of C126, H302 and N337. Several functional amino acid residues are hypothesized from crystal structure: 1) E154, which is proposed to initiate the enzymatic reaction. 2) It is proposed that S340 forms a hydrogen bond network in reaction process.

2.2 Enzyme assay of StlD mutants

To attain the functional information of important amino acid residues, many mutants were constructed. C126A, C126S, C126D, H302R, N337A, N337Q, E154A, E154D, E154Q, S340A, S340T, and S340D completely lost the activtiy. This result demonstrates that StlD may be very sensitive for mutation because of its small catalytic cavity. Interestingly, StlD H302A, a conserved residue of catalytic triad in KSs, maintained comparable activity with WT StlD. This result reveals that conserved H302 losts its function in decarboxylation or binding the intermediate in the catalytic process.

2.3 Enzymatic synthesis of novel compounds using unnatural substrate

For analysis of the substrate diversity of StlD and StlC, unnatural substrates alkyl β-ketoacyl-SNACs were used. Several novel stilbene analogues were detected. Therefore, it is shown that StlD and StlC both exhibit the tolerance toward unnatural substrate.

Conclusion

In this study, two unusual non-decarboxylative condensation enzymes were studied using structural basis and precursor-directed approach. The results include structural basis, catalytic mechanism and enzymatic synthesis as following.

1. Structural basis: high-resolution crystal structures of two non-decarboxylative condensation enzymes are determined, including WT CsyB, mutants CsyB I375F, CsyB I375W, WT StlD, and StlD H302A. On the basis of crystal structure, it is demonstrated that CsyB catalyzes the decarboxylative condensation as other type III PKS owing to its conversed type III PKS catalytic triad, while its non-decarboxylative condensation depends on the introduction of two vital amino acids H377 and I375. In addition, it is suggested that a CsyB-specific novel cavity accommodates the branching group of substrate. On the other hand, StlD performs the non-decarboxylative condensation that activate by E154 base on the crystal structure.

2. Catalytic mechanism: 1) activation mode: H377 is responsible for activation C_2 position of β-ketoacyl thioester in CsyB; whereas E154 is responsible for activate the C_4 position of β-ketoacyl thioester in StlD. 2) function of hydrogen bond network: hydrogen bond network releases the intermediate in CsyB, while it releases the final product in StlD. In addition, in CsyB, I375 controls the volume of novel cavity.

3. Enzymatic synthesis: two types of novel compounds, α-pyrone and stilbene analogues, were enzymatically synthesized using unnatural substrates by CsyB and StlD, respectively.