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Structure and mechanism of chalcone synthase-like polyketide synthases

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Polyketide synthases (PKS) produce an array of natural products with different biological activities and pharmacological properties by varying the starter and extender molecules that form the final polyketide. Recent studies of the simplest PKS, the chalcone synthase (CHS)-like enzymes involved in the biosynthesis of flavonoids, anthocyanin pigments, and antimicrobial phytoalexins, have yielded insight on the molecular basis of this biosynthetic versatility. Understanding the structure–function relationship in these PKS provides a foundation for manipulating polyketide formation and suggests strategies for further increasing the scope of polyketide biosynthetic diversity. *Journal of Industrial Microbiology & Biotechnology* (2001) **27,** 393–398.

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Introduction

Polyketide synthases (PKS) catalyze the biosynthesis of structurally diverse natural products in plants, fungi, and bacteria [9,18,22]. These natural products exhibit different biological activities and pharmacological properties, including antimicrobial, immunosuppressant, and anticancer uses. PKS generate a polyketide chain from simple molecular building blocks, like acetate and propionate units, by catalyzing a series of decarboxylative condensation reactions. The amazing structural diversity of these molecules results from varying the length and constituents of the polyketide and through the enzymes that modify the final scaffold. In plants, exploitation of polyketide biosynthetic versatility offers new avenues for improving disease resistance, micronutrient incorporation into human crops, and production of pharmaceuticals in transgenic plants [3,8].

The chalcone synthase (CHS)-like PKS are structurally and mechanistically the simplest PKS [18,22]. These enzymes, also known as type III PKS [23], function as homodimeric iterative PKS (monomer $M_r \approx 42-45$ kDa) with two independent active sites that catalyze a series of decarboxylation, condensation, and cyclization reactions [26]. Recent molecular cloning and biochemical studies establish this class of PKS as a widespread enzyme family in plants and bacteria that catalyzes a diverse set of biosynthetic reactions [2,5,7,20]. The best studied type III PKS is CHS. Physiologically, CHS is essential for formation of 4,2′,4′,6′-tetrahydroxychalcone (chalcone; Figure 1), an essential secondary metabolite in the biosynthesis of anthocyanin pigments, antimicrobial phytoalexins, and flavonoid inducers of *Rhizobium* nodulation genes [4,15].

Structural and functional studies of *Medicago sativa* (alfalfa) CHS2 and the homologous 2-pyrone synthase (2-PS) from *Gerbera hybrida* (daisy) provide a view of the catalytic machinery involved in polyketide assembly and elucidate the structural basis for control of polyketide length in type III PKSs. With the structural and mechanistic information currently available, steps toward re-engineering polyketide formation in these PKS are the next goal.

Structure of CHS and its relationship to other condensing enzymes

The 1.56 Å-resolution crystal structure of alfalfa CHS2 [6] reveals that the enzyme forms a symmetric dimer and provides a structural archetype for the type III PKS (Figure 2). The dimer interface is a fairly flat surface delineated by two structural features. First, the N-terminal α-helix of monomer A entwines with the corresponding α-helix of monomer B. Second, a tight loop containing a *cis*-peptide bond between Met137 and Pro138 exposes the methionine side chain as a knob on the monomer surface. Across the interface, Met137 protrudes into a hole found in the surface of the adjoining monomer to form part of the active site cavity. Thus, dimerization forms the complete CHS active site.

Structures of CHS complexed with different coenzyme A (CoA) thioesters and product analogs (i.e., naringenin and resveratrol) show that the active site is buried within an interior cavity located at the cleft between the upper and lower domains of each monomer (Figure 2) [6]. Considering the complexity of the reaction mechanism leading to chalcone formation, there are remarkably few chemically reactive amino acids in the active site. In particular, four residues conserved in the known CHS-related enzymes (Cys164, Phe215, His303, and Asn336) define the catalytic machinery of CHS (described below). Access to the active site cavity is gained through a 16 Å-long tunnel that forms the CoA binding site. Structures of CHS complexed with CoA, acetyl-CoA, and hexanoyl-CoA reveal that the pantetheine arm of each ligand extends through the tunnel to position the thioester-linked substrates near the active site cysteine [6].

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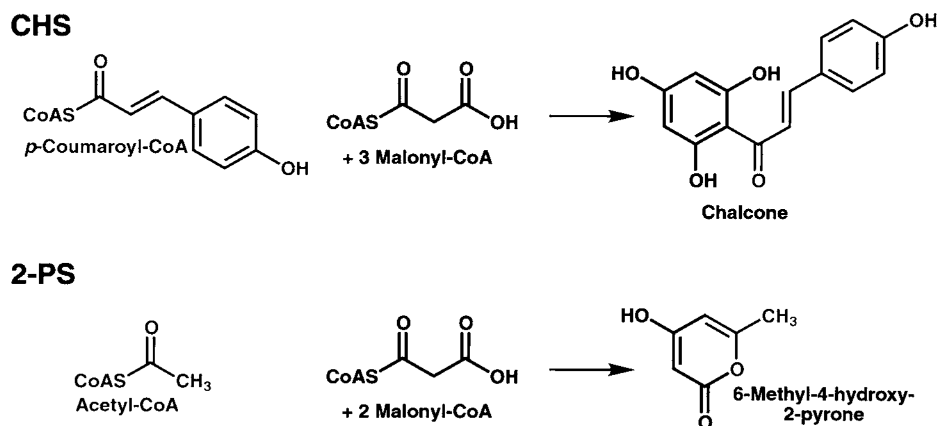

Figure 1 Overall reaction of CHS and 2-PS.

Each CHS monomer consists of two structural domains (Figure 3a, left). The upper domain exhibits the $\alpha-\beta-\alpha-\beta-\alpha$ pseudo-symmetric motif observed in fatty acid β-ketoacyl synthase (Figure 3a, right) [10]. Both CHS and β-ketoacyl synthase use a cysteine as a nucleophile in the condensation reaction and shuttle reaction intermediates *via* CoA thioester-linked molecules or acyl carrier proteins (ACP), respectively. The conserved architecture of the upper domain maintains the three-dimensional position of the catalytic residues of each enzyme (Cys164, His303, and Asn336 in CHS correspond to Cys163, His303, and His340 in β-ketoacyl synthase). These catalytic residues are also conserved in the sequences of the ketosynthase domains from modular and aromatic PKSs (Figure 3b). Structural differences in the lower domain of CHS create a larger active site cavity than that of β-ketoacyl synthase and provide space for the tetraketide required for chalcone formation from p-coumaroyl-CoA and three malonyl-CoAs. In contrast, the active site of β-ketoacyl synthase catalyzes the condensation reaction that elongates palmoleitic acid (C16:1) by a single acetate unit to *cis*-vaccenic acid (C18:1). The similar structural features and chemistry of these enzymes imply a common evolutionary origin for the CHS-like enzymes and the ketosynthases involved in fatty acid and polyketide biosynthesis.

Mechanism of polyketide formation in CHS

CHS orchestrates the condensation of one p-coumaroyl-CoA and three malonyl-CoA molecules into chalcone [14] (Figure 1). Transfer of the p-coumaroyl moiety from the CoA-linked starter molecule to Cys164 at the active site initiates the reaction mechanism. Next, the sequential condensation of three acetate units derived from three malonyl-CoA molecules with the enzyme-bound coumaroyl moiety forms a tetraketide intermediate. Inherent in the condensation reaction is decarboxylation of

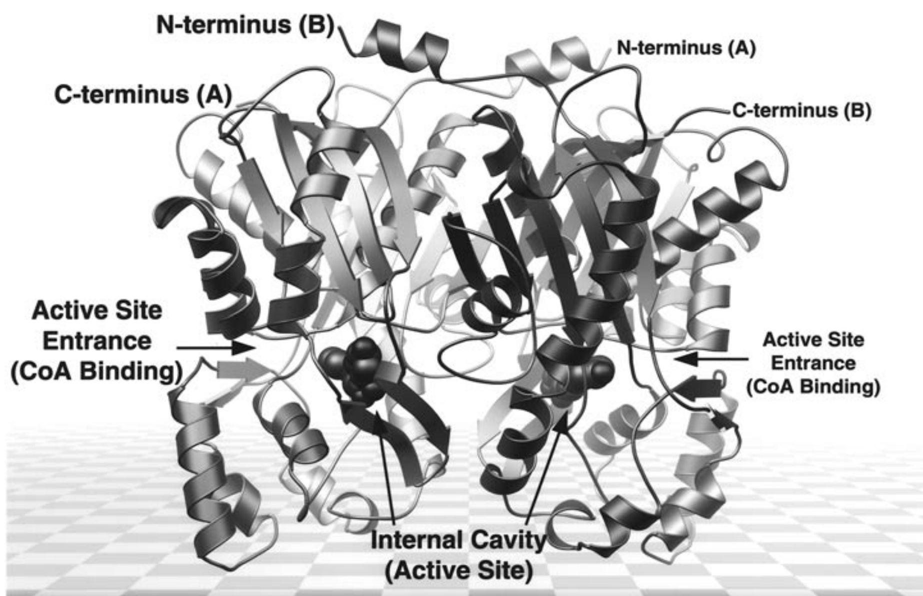

Figure 2 Overall CHS-like fold. The N- and C-termini for each monomer are indicated. The position of the active site cavity is indicated by the position of the bound naringenin molecule (shown as a space-filling model). The CoA-binding tunnel provides access to the internal cavity.

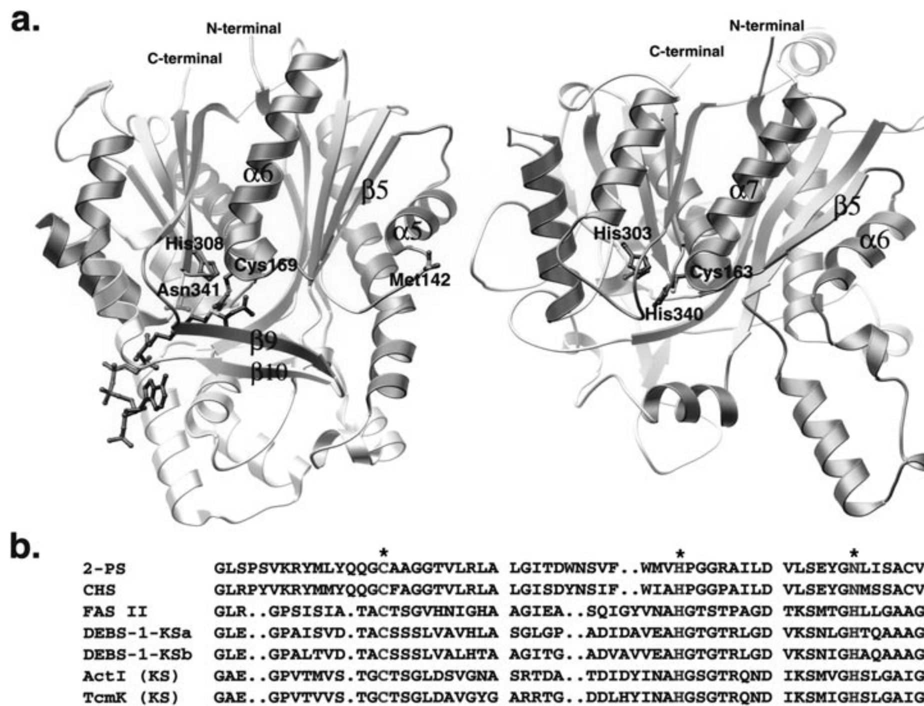

Figure 3 (a) Comparison of the CHS monomer (left) and β-ketoacyl synthase monomer (right). The side chains of the catalytic residues of CHS (Cys164, His303, Asn336) and β-ketoacyl synthase (Cys163, His303, His340) are shown. (b) Sequence conservation of the catalytic residues of CHS, 2-PS, β-ketoacyl synthase (FAS II), and the ketosynthase modules of 6-deoxyerythronolide B synthase (DEBS), actinorhodin synthase (ActI) and tetracenomycin synthase (TcmK). The catalytic residues are highlighted with an *.

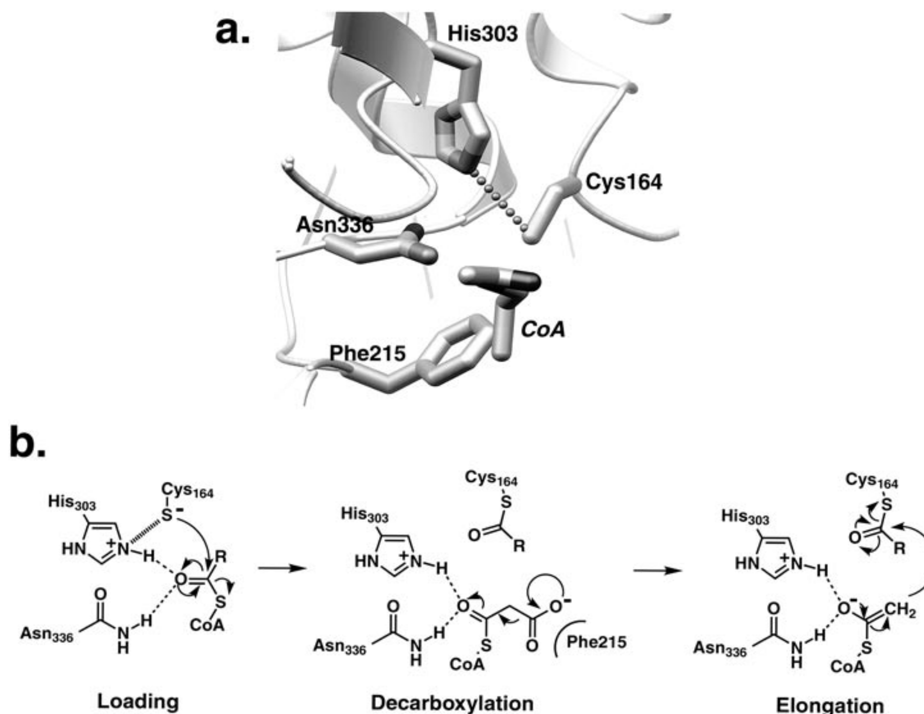

Figure 4 CHS active site and reaction mechanism. (a) The catalytic residues are shown along with the terminal end of a CoA molecule. The ionic interaction between Cys164 and His303 is indicated by the dotted line. (b) Proposed reaction mechanism of CHS. The loading, decarboxylation, and elongation steps are shown. R is the coumaroyl moiety in the first reaction cycle, coumaroyl-acetyl group in the second cycle, and a coumaroyl-diacetyl group in the final cycle.

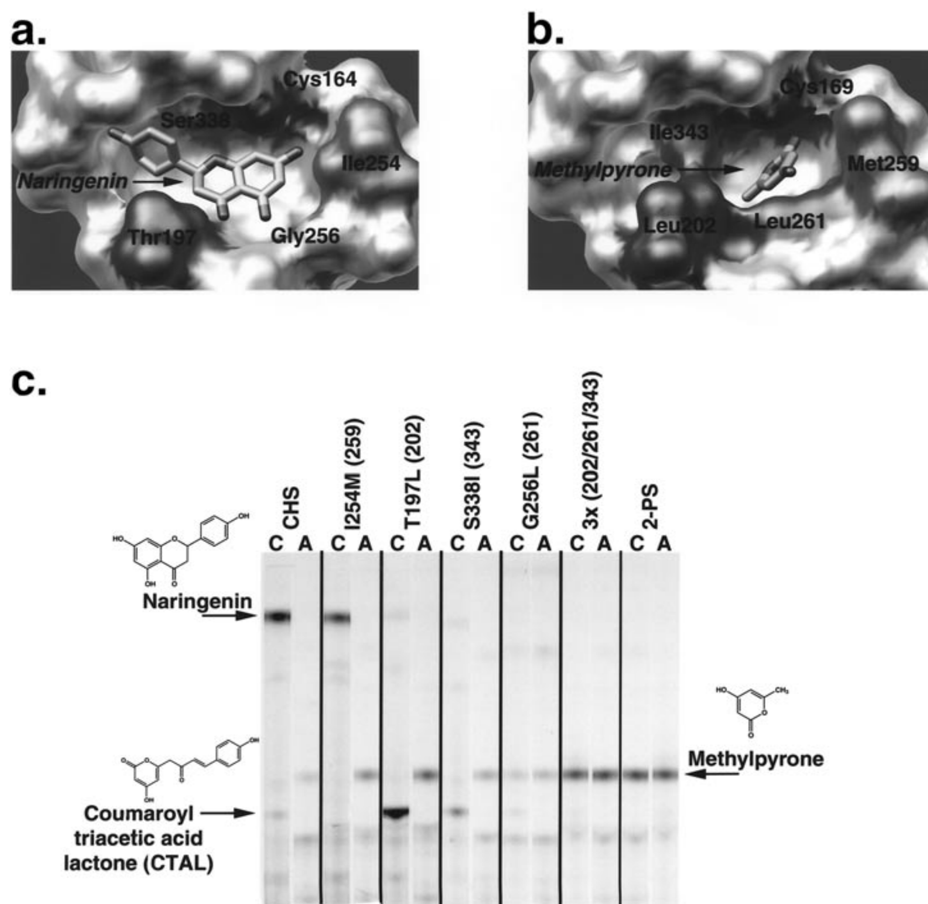

Figure 5 Size of the initiation/elongation pocket dictates polyketide formation. Surface representations of the CHS (a) and 2-PS (b) active site cavities are shown. The CHS·naringenin complex structure was previously determined [14] and the 2-PS·methylpyrone complex is modeled for comparison. The catalytic cysteines, the three positions that convert CHS into 2-PS, and the substitution that does not affect product formation are highlighted and labelled. (c) TLC analysis of CHS, 2-PS, and CHS mutant enzymes. The radiogram shows the radiolabeled products produced by incubation of each protein with [^{14}C]malonyl-CoA and either *p*-coumaroyl-CoA (c) or acetyl-CoA (a). Numbering of mutants corresponds to CHS with 2-PS numbering in parenthesis. Positions of reaction products and their identities are indicated.

malonyl-CoA to an acetyl-CoA carbanion that serves as a nucleophile during the chain-elongation reactions. Four amino acids (Cys164, Phe215, His303, and Asn336) situated at the intersection of the CoA-binding tunnel and the active site cavity play essential and distinct roles during malonyl-CoA decarboxylation and chalcone formation (Figure 4a). A series of functional studies focusing on the properties of site-directed mutants of these residues has examined the reaction mechanism of CHS [11,13,16,25].

In the initial loading reaction, the thiolate nucleophile of Cys164 attacks the thioester carbonyl, resulting in transfer of the coumaroyl moiety to the cysteine side chain (Figure 4b, R=coumaroyl) [13,16]. The thiolate anion of Cys164 is maintained by an ionic interaction with the imidazolium cation of His303 [11,25]. His303 and Asn336 hydrogen bond with the thioester carbonyl, further stabilizing formation of the tetrahedral reaction intermediate. CoA dissociates from the enzyme, leaving a coumaroyl-thioester at Cys164.

Next, malonyl-CoA binds and positions the bridging carbon of the malonyl moiety near the carbonyl of the enzyme-bound coumaroyl thioester. Asn336 orients the thioester carbonyl of malonyl-CoA near His303 with Phe215 providing a nonpolar environment for the terminal carboxylate that facilitates decarboxylation [13]. His303 and Asn336 interact with the substrate's thioester carbonyl, creating an efficient electron sink or oxyanion hole that stabilizes the developing negative charge during the decarboxylation step through stabilization of the enol tautomer of the acetyl anion. Moreover, the presence of a stabilized anion also reduces its reactivity toward CO_2, driving the decarboxylation reaction forward.

In the elongation step, attack of the carbanion on the carbonyl of the enzyme-bound coumaroyl thioester releases the thiolate anion of Cys164 and transfers the coumaroyl group to the acetyl moiety of the CoA thioester. Hydrogen bonds from His303 and Asn336 stabilize the tetrahedral transition state of this reaction. Recapture of the elongated coumaroyl-acetyl-diketide-CoA by Cys164 and release of CoA set the stage for two additional rounds of malonyl-CoA decarboxylation and elongation, resulting in formation of the final tetraketide reaction intermediate.

The final step in chalcone formation involves an intramolecular Claisen condensation encompassing the three acetate units derived from three malonyl-CoAs. During cyclization, the nucleophilic methylene group nearest the coumaroyl moiety attacks the carbonyl carbon of the thioester linked to Cys164. Ring closure is proposed

to proceed through an internal proton transfer from the nucleophilic carbon to the carbonyl oxygen. Breakdown of this tetrahedral intermediate expels the newly cyclized ring system from Cys164. Subsequent aromatization of the trione ring through a second series of facile internal proton transfers yields chalcone.

Specificity of polyketide formation: starter molecule selection, polyketide length, and cyclization reaction

The type III PKS generate molecular diversity in their products by selection of different starter molecules, by varying the length of the polyketide chain, and through regiospecific control of the polyketide cyclization reaction. Elucidating how type III PKS achieve specificity in polyketide formation is essential for manipulating the activities of these enzymes.

The CHS-like PKS utilize a wide range of starter molecules [18]; however, the substrate specificity of individual members of this enzyme family also varies greatly. For example, *in vitro* CHS accepts an array of aromatic and aliphatic starter molecules with different catalytic efficiencies to yield assorted phloroglucinol and lactone products [21,27]. Recent work capitalizing on the permissive substrate specificity of CHS demonstrates formation of unnatural polyketides from synthetic cinnamoyl-CoA analogs and alternative aromatic CoA starter molecules [1,17]. Previous mutagenesis experiments with stilbene synthase, a CHS-like PKS, indicate that single point mutations also alter starter molecule preference [19]. Currently, a systematic examination of how mutations in the active site cavity affects polyketide formation is in the early stages. Recently, alfalfa CHS2 was converted into 2-pyrone synthase (2-PS) by site-directed mutagenesis [12].

Alfalfa CHS2 and 2-PS from *Gerbera hybrida* are related by 74% amino acid identity, but 2-PS forms the triketide methylpyrone from an acetyl-CoA starter molecule and two malonyl-CoAs [5] (Figure 1). Together CHS and 2-PS provide a comparative system for elucidating the structural features that govern starter molecule selectivity and the final length of the polyketide product.

The three-dimensional structures of 2-PS and CHS are nearly identical and superimpose with an r.m.s. deviation of 0.64 Å for the two proteins' C_α atoms [12]. The catalytic residues and the CoA binding sites of both proteins are structurally conserved. Interestingly, comparison of the three-dimensional structures of 2-PS and CHS reveals that the volume of the active site cavity in each protein is dramatically different (Figure 5a and b). The 2-PS cavity is approximately one third the size of the CHS cavity, implying that the volume of the active site cavity influences starter molecule selectivity and limits polyketide length between the two PKSs.

To demonstrate this principle, the active site cavity of CHS was modified to resemble that of 2-PS by site-directed mutagenesis [12]. Kinetic characterization and identification of reaction products confirmed that a combination of three amino acid substitutions (T197L/G256L/S338I) in CHS changed starter molecule preference from *p*-coumaroyl-CoA to acetyl-CoA and resulted in formation of a triketide instead of a tetraketide product (Figure 5c). Surprisingly, introduction of each substitution as a single mutation prevented chalcone formation but did not interfere with generation of the tetraketide intermediate, since the reaction product was identified as coumaroyltriacetic acid lactone (CTAL). In these mutants, the regiospecific cyclization reaction of the tetraketide was derailed. These experiments and those of Suh *et al* [24] demonstrate that substitutions of residues lining the active site cavity can derail the intramolecular Claisen condensation reaction to a nonspecific lactonization of the polyketide intermediate.

Conclusions

The three-dimensional structures of CHS and 2-PS combined with mechanistic insights on how these enzymes assemble polyketides provide a starting point for experiments aimed at manipulating polyketide biosynthesis in the plant and bacterial type III PKSs. Initial studies have demonstrated that these PKSs possess a relaxed substrate specificity. Together with engineered variants that accept unnatural starter molecules, catalyze a different number of elongation steps, or exhibit altered cyclization reactions, the potential for expanding the diversity of molecular scaffolds generated by the CHS-like PKSs is only just beginning to be explored.

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