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Enzymatic formation of long-chain polyketide pyrones by plant type III polyketide synthases

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Abstract

Recombinant chalcone synthase (CHS) from *Scutellaria baicalensis* and stilbene synthase (STS) from *Arachis hypogaea* accepted CoA esters of long-chain fatty acid (CHS up to the C_{12} ester, while STS up to the C_{14} ester) as a starter substrate, and carried out sequential condensations with malonyl-CoA, leading to formation of triketide and tetraketide α-pyrones. Interestingly, the C_6, C_8, and C_{10} esters were kinetically favored by the enzymes over the physiological starter substrate; the k_{cat}/K_M values were 1.2- to 1.9-fold higher than that of *p*-coumaroyl-CoA. The catalytic diversities of the enzymes provided further mechanistic insights into the type III PKS reactions, and suggested involvement of the CHS-superfamily enzymes in the biosynthesis of long-chain alkyl polyphenols such as urushiol and ginkgolic acid in plants.

Keywords: Chalcone synthase; Stilbene synthase; Type III polyketide synthase; Long-chain α-pyrones; Urushiol; Ginkgolic acid

1. Introduction

The chalcone synthase (CHS) superfamily of type III polyketide synthases (PKSs) are pivotal enzymes in the biosynthesis of flavonoids as well as a wide range of structurally diverse, biologically important natural products (Schröder, 1999; Austin and Noel, 2003). The homodimer of relatively modest-sized proteins of 40–45 kDa, sharing 25–90% amino acid sequence identity with each other, catalyze the assembly of complex natural products by successive decarboxylative condensations of malonyl-CoA in a biosynthetic process that closely parallels fatty acid biosynthesis. CHS (EC 2.3.1.74) and stilbene synthase (STS) (EC 2.3.1.95) thus perform sequential condensation of the C_6–C_3 unit of *p*-coumaroyl-CoA (**1**) as a starter with three C_2 units from malonyl-CoA (**2**). After three rounds of the polyketide chain elongation reaction, cyclization of the enzyme-bound tetraketide intermediate lead to formation of 4,2′,4′,6′-tetrahydroxychalcone (naringenin chalcone) (**3**) or *trans*-3,4′,5-trihydroxystilbene (resveratrol) (**4**), respectively (Scheme 1). In addition, in enzyme reactions in vitro, a triketide and a tetraketide α-pyrone; bisnoryangonin (BNY) (**5**) (Kreuzaler and Hahlbrock, 1975a,b) and *p*-coumaroyltriacetic acid lactone (CTAL) (**6**) (Akiyama et al., 1999), are also obtained as early released derailment by-products. Crystallographic and site-directed mutagenesis studies on alfalfa (*Medicago sativa*) CHS revealed the active site machinery of the chalcone forming reaction which proceeds through starter molecule loading at Cys164, malonyl-CoA decarboxylation, polyketide chain elongation, followed by cyclization and aromatization of the enzyme-bound tetraketide intermediate (Ferrer et al., 1999; Jez and Noel, 2000; Jez et al., 2000a,b, 2001, 2002; Tropf et al., 1995; Suh et al., 2000a,b; Abe et al., 2001, 2003a,b). The active-site of the enzyme is composed of the

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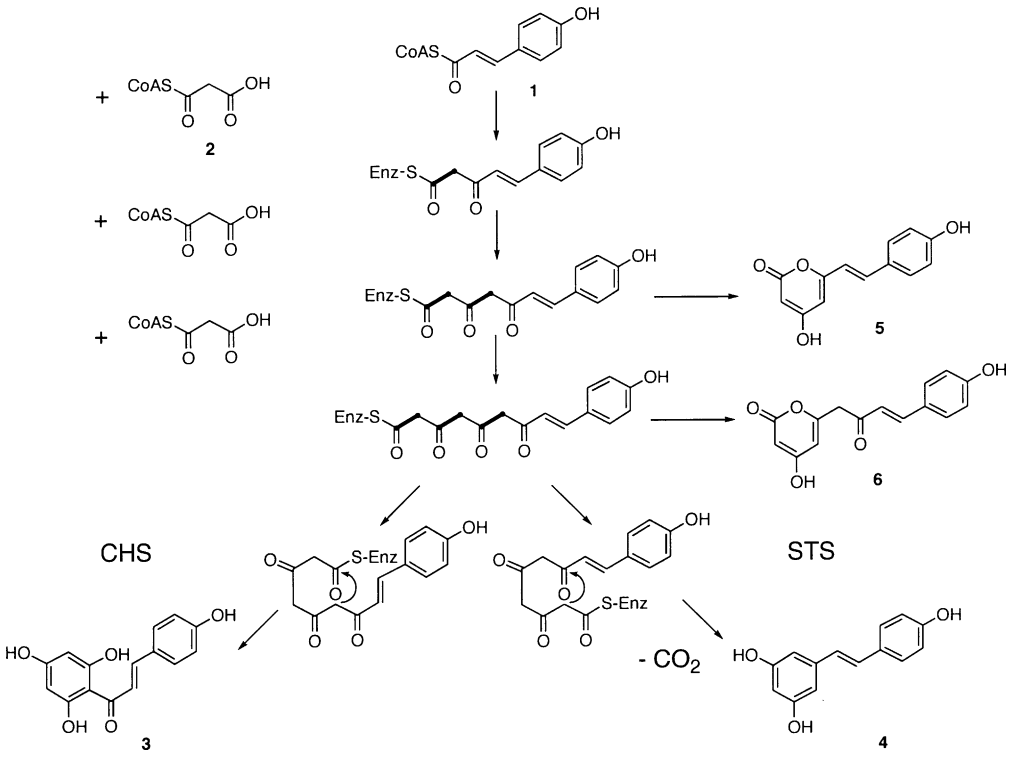
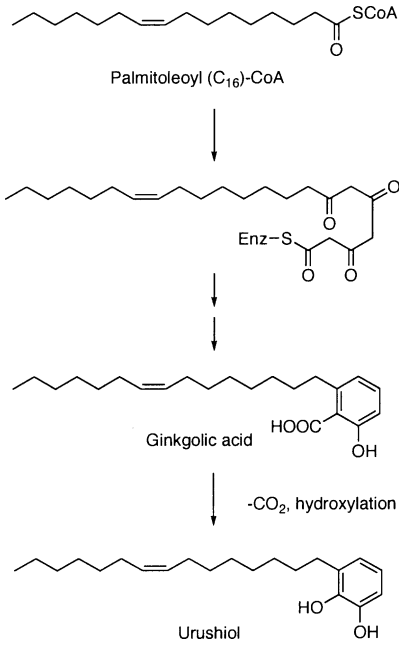


Scheme 1. Proposed mechanism for the conversion of *p*-coumaroyl-CoA (**1**) and malonyl-CoA (**2**) to naringenin chalcone (**3**) and resveratrol (**4**) by CHS and STS. The pyrone derivatives, bisnoryangonin (BNY) (**5**) and 4-coumaroyltriacetic acid lactone (CTAL) (**6**) are common by-products of the enzyme reactions in vitro.

coumaroyl-binding pocket and the cyclization pocket, defined by four residues conserved in the type III PKS enzymes (Cys164, Phe215, His303, and Asn336).

The functional diversity and the promiscuity of the CHS-superfamily enzymes are remarkable. In previous studies, we demonstrated that recombinant CHS from *Scutellaria baicalensis* (Labiatae) and STS from *Arachis hypogaea* (Fabaceae) have unusually broad substrate specificities toward the starter and the extender substrate (Abe et al., 2000, 2001, 2002, 2003a,b; Morita et al., 2000, 2001). Thus, instead of *p*-coumaroyl-CoA, the enzymes accepted a variety of aromatic and aliphatic CoA esters as a starter substrate, and efficiently yielded a series of chemically and structurally different unnatural polyketides. Furthermore, both CHS and STS also accepted methylmalonyl-CoA as an extension substrate to catalyze formation of an unnatural C_6–C_5 aromatic polyketide. The enzymes even afforded unnatural polyketides when both the starter and the extender substrate were simultaneously replaced with non-physiological analogues.

It has been postulated that CoA esters of long-chain fatty acid such as palmitoleoyl (C_{16})–CoA can act as a starter substrate for malonyl-CoA chain extension, leading to formation of alkyl polyphenols including urushiol and ginkgolic acid (anacardic acid), the allergic substances of lacquer tree (*Rhus verniciflua*, Anacardiaceae) and ginkgo tree (*Ginkgo biloba*, Ginkgoaceae), respectively (Scheme 2) (Dewick, 2002). Further, it was recently reported that a mycobacterial type III PKS



Scheme 2. Proposed mechanism for the biosynthesis of urushiol and ginkgolic acid (anacardic acid) from palmitoleoyl-CoA.

from *Mycobacterium tuberculosis*, sharing 25–45% amino acid sequence similarity with plant CHSs, catalyzed formation of triketide and tetraketide α-pyrones from long-chain fatty acyl-CoA (C_{12}–C_{20}) (Saxena et al., 2003). These suggest possible involvement of the type III CHS-superfamily enzymes also in the biosynthesis of the alkyl polyphenols in plants. Considering the broad substrate specificities of CHS and STS, it was thus interesting to test whether the enzymes also accept the CoA-esters of long-chain fatty acid as a starter of the polyketide formation reactions. Here in this paper, we describe enzymatic conversion of long-chain fatty acyl-CoAs (C_6–C_{18}) by recombinant *S. baicalensis* CHS and *A. hypogaea* STS. Interestingly, the C_6, C_8, and C_{10} esters were kinetically favored by the type III CHS-superfamily enzymes over the physiological starter *p*-coumaroyl-CoA.

2. Results and discussion

The TLC based assay as well as the LC-ESIMS analyses of the enzyme reaction products revealed that both recombinant *S. baicalensis* CHS and *A. hypogaea* STS readily accepted the CoA esters of long-chain fatty acid (**7a**–**7e**) as a starter substrate for the polyketide chain elongation reactions (Fig. 1). *S. baicalensis* CHS accepted the C_6–C_{12} ester, while *A. hypogaea* STS up to the C_{14} ester, and carried out sequential decarboxylative condensations with malonyl-CoA to yield triketide α-pyrones (**8a**–**8e**) as major products. In this regard, the STS showed broader substrate tolerance toward the unnatural substrates than the CHS. Formations of tetraketide α-pyrones (**9a**–**9e**) were also detected, however, resorcinol (1,3-dihydroxybenzene) (**10**) or phloroglucinol (1,3,5-trihydroxybenzene) (**11**) derivatives with a newly formed aromatic ring system (Scheme 3) were not detected in the reaction mixtures.

The structure of the enzyme reaction products were unambiguously determined by UV and LC-ESIMS, using 4-hydroxy-6-pentyl-2-pyrone (**8a**) and 4-hydroxy-6-(2-oxoheptyl)-2-pyrone (**9a**) obtained in our previous works, as authentic compounds (Morita et al., 2000, 2001). Each enzyme reaction product showed a UV spectrum similar to either that of **8a** or **9a**. Further, the LC-ESIMS spectrum gave a parent ion peak $[M - H]^-$ indicating the polyketide formation reaction had terminated after two (triketide lactone) or three (tetraketide lactone) condensations of malonyl-CoA. Moreover, in MS/MS, the fragment corresponding to $[M - H–CO_2]^-$ confirmed the presence of a α-pyrone ring structure. The enzyme reactions were therefore terminated without aromatic ring formation. Either an acid catalyzed lactonization of linear derailment products in solution, or the acid stabilization of lactones originally formed within the active site of the enzyme, resulted in the formation of the triketide and tetraketide α-pyrones.

In earlier work, it has been reported that the parsley (*Petroselinum horstense*) CHS accepted butyryl (C_4)-CoA and hexanoyl (C_6)-CoA as starter substrates in pH dependent manner; formation of phloroglucinol derivatives from these substrates was only observed at pH 6.5 (Schüz et al., 1983), but not at pH 8.0, the optimum pH of the enzyme for chalcone formation from the physiological starter *p*-coumaroyl-CoA (Kreuzaler and Hahlbrock, 1975a,b). Presumably, the pH change significantly affected charge distribution in the enzyme–substrate and enzyme–intermediate complex, including the most important charged interactions of the catalytic triad of Cys164, His303, and Asn336, thus greatly

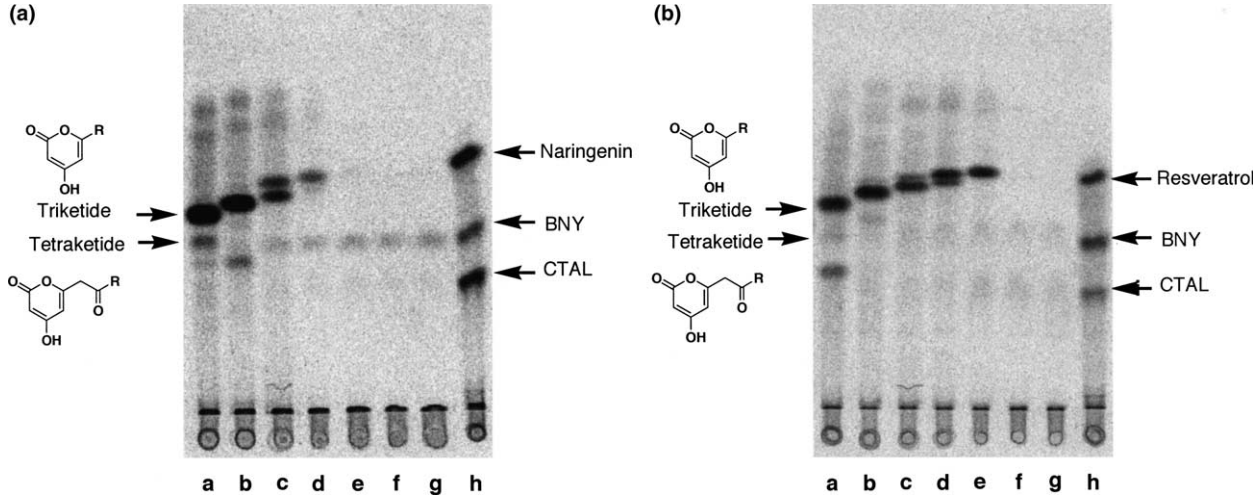
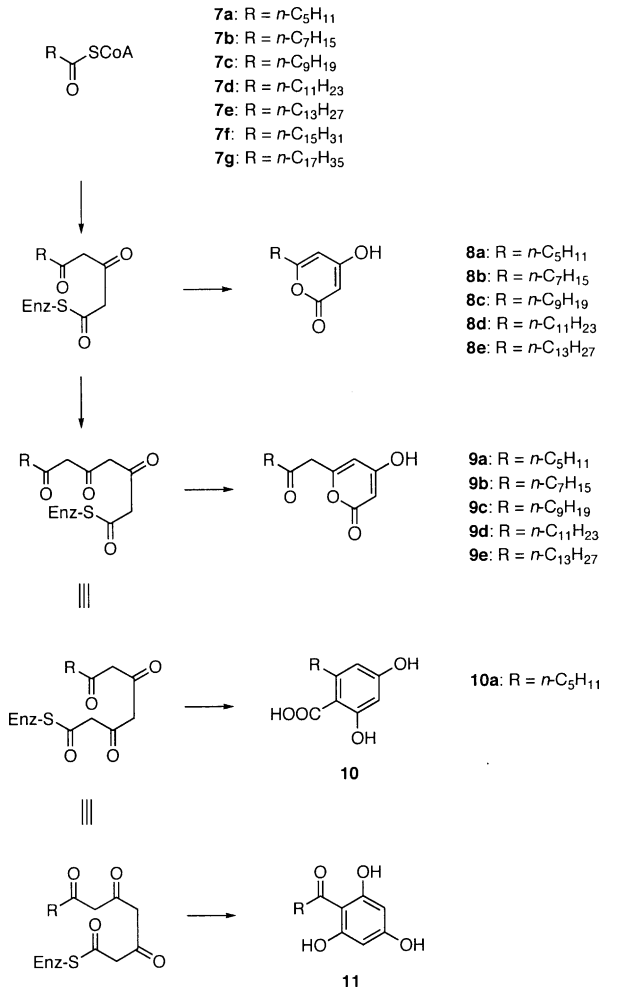


Fig. 1. TLC based analysis of radiolabeled products of CoA-esters of long-chain fatty acid (**7a**–**7g**) by: (a) *S. baicalensis* CHS and (b) *A. hypogaea* STS. Lane a, hexanoyl (C_6)-CoA; lane b, octanoyl (C_8)-CoA; lane c, decanoyl (C_{10})-CoA; lane d, dodecanoyl (C_{12})-CoA; lane e, tetradecanoyl (C_{14})-CoA; lane f, hexadecanoyl (C_{16})-CoA; lane g, octadecanoyl (C_{18})-CoA; lane h, *p*-Coumaroyl-CoA.



Scheme 3. Enzymatic conversion of long-chain acyl-CoAs (**7a**–**7g**) by recombinant *S. baicalensis* CHS and *A. hypogaea* STS. Resorcinol derivatives (**10**) and (**11**) with a newly formed aromatic ring system were not detected in the reaction mixtures.

influenced the chain elongation reactions of CHS-superfamily enzymes. In the case of the reactions with the long-chain acyl-CoAs, the triketide pyrone forming activities of CHS were maximum at pH 8.0 in Tris–HCl buffer, while those of STS exhibited fairly broad pH optimum within a range of pH 7.0–9.0 in either Tris–HCl or potassium phosphate buffer (Fig. 2). On the other hand, the tetraketide pyrone forming enzyme activities were slightly higher under acidic pH, which parallels with the previous observation that most of *S. baicalensis* CHS mutants efficiently catalyzed increased number of condensation reactions under acidic condition (Abe et al., 2003b).

Interestingly, steady-state kinetic analysis revealed that the C_6, C_8, and C_{10} ester were apparently even better starter substrates for both CHS and STS than the physiological starter *p*-coumaroyl-CoA (Table 1). In particular for *S. baicalensis* CHS, the k_{cat} values of the C_6, C_8, and C_{10} esters (**7a**–**7c**) for the "triketide" pyrone formation were 2.7- to 8.8-fold higher than that of *p*-coumaroyl-CoA while the k_{cat}/K_M values were 1.2- to 1.9-fold higher than that of the normal substrate. The enzyme reaction rates were thus significantly increased when compared with that for the formation of the aromatic tetraketides, chalcone or stilbene, from *p*-coumaroyl-CoA. In contrast, it has been reported that medium chain aliphatic CoAs (C_6–C_8) were poor substrates for recombinant *M. sativa* CHS; the k_{cat}/K_M values were 0.7- to 0.9-fold decreased compared with that of *p*-coumaroyl-CoA (Jez et al., 2002). This may suggest species difference between *S. baicalensis* (Labiatae) and *M. sativa* (Leguminosae) in the active site structure of the CHS enzyme.

It was demonstrated for the first time that the plant CHS-superfamily enzymes accepted the CoA esters of

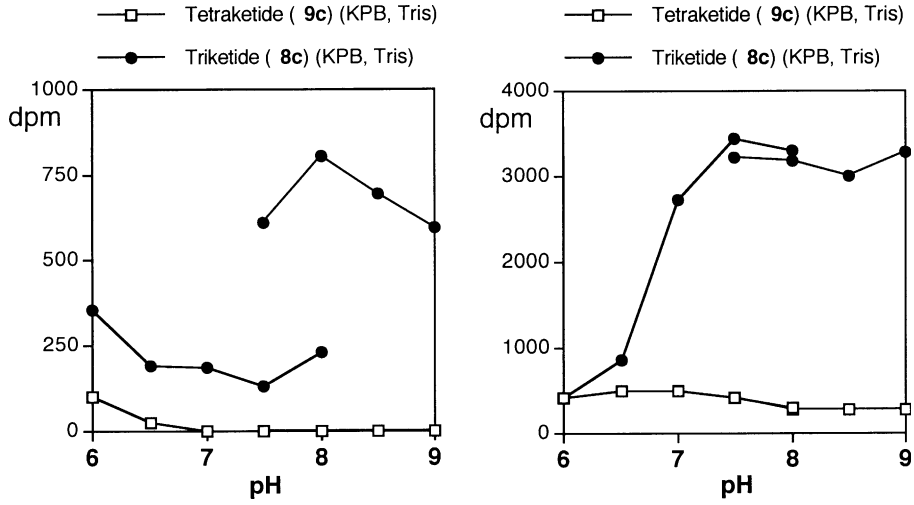


Fig. 2. The pH dependence of enzyme activities of: (A) *S. baicalensis* CHS and (b) *A. hypogaea* STS when decanoyl (C_{10})-CoA (**7c**) was used as a starter substrate.

Table 1
Steady-state kinetic parameters for enzyme reactions[a]

Starter substrate	K_{cat} (min^{-1})	K_M (μM)	k_{cat}/K_M ($s^{-1}\ M^{-1}$)
S. baicalensis CHS			
p-Coumaroyl-CoA	1.00 ± 0.07	5.17 ± 2.62	3224
Hexanoyl (C_6)-CoA	8.82 ± 1.08	24.50 ± 9.87	6000
Octanoyl (C_8)-CoA	3.97 ± 0.24	12.19 ± 1.98	5428
Decanoyl (C_{10})-CoA	2.65 ± 0.44	11.04 ± 3.48	4001
A. hypogaea STS			
p-Coumaroyl-CoA	0.65 ± 0.09	5.11 ± 2.14	2120
Hexanoyl (C_6)-CoA	1.01 ± 0.11	7.78 ± 2.71	2164
Octanoyl (C_8)-CoA	2.08 ± 0.11	11.70 ± 1.31	2963
Decanoyl (C_{10})-CoA	1.43 ± 0.10	4.17 ± 1.05	5715
Dodecanoyl (C_{12})-CoA	0.50 ± 0.12	5.74 ± 0.26	1451

[a] Steady-state kinetic parameters were calculated for formation of major product of the enzyme reaction, i.e. triketide α-pyrones, except for those of *p*-coumaroyl-CoA which was for formation of naringenin chalcone. Lineweaver–Burk plots of data were employed to derive the apparent K_M and k_{cat} values (average of triplicates ± standard deviation) using EnzFitter software (BIOSOFT).

long-chain fatty acid (up to C_{14}) as a starter substrate and carried out sequential decarboxylative condensations with malonyl-CoA, leading to formation of triketide and tetraketide α-pyrones. The unusually broad substrate specificities and the catalytic diversities of the enzymes provided further mechanistic and stereochemical insights into the polyketide formation reactions of the type III PKS enzymes. Notably, the results suggested possible involvement of the CHS-superfamily enzymes also in the biosynthesis of long-chain alkyl polyphenols including the palmitoleoyl (C_{16})-CoA derived urushiol and ginkgolic acid (anacardic acid), the allergic substances of lacquer tree (*R. vernicifiua*, Anacardiaceae) and ginkgo tree (*G. biloba*, Ginkgoaceae), respectively (Scheme 2) (Dewick, 2002) although the formation of these metabolites still needs aromatization of the enzyme-bound tetraketide intermediate. None of the responsible enzymes involved in the biogenesis of the long-chain alkyl polyphenols have been identified yet. On the other hand, a novel plant-specific type III PKS catalyzing formation of olivetolic acid (**10a**) from a medium-chain hexanoyl (C_6)-CoA as a starter substrate (Scheme 3) has been recently cloned from *Cannabis sativa* (Cannabinaceae), and regarded as a key enzyme in the biosynthesis of the cannabinoids (Tanaka et al., 2001).

Finally, it should be noted that the active site cavity of either CHS or STS is not apparently large enough to accommodate the α-pyrones with the long aliphatic side chain, suggesting the possibility of an altered binding pocket as in the case of the recently reported mycobacterial type III PKS from *M. tuberculosis* (Saxena et al., 2003). Interestingly, Gokhale and co-workers proposed a presence of an additional hydrophobic acyl-binding pocket based on the structural similarity between the *M. tuberculosis* PKS and β-ketoacyl synthase (KAS III) that uses long aliphatic chains as substrates (Saxena et al., 2003). Both enzymes share the Cys-His-Asn catalytic triad as well as the thiolase αβαβα overall fold architecture that forms one side of the active site cavity (Austin and Noel, 2003). Manipulation of the CHS-superfamily of the type III PKS enzyme reactions would thus lead to further production of chemically and structurally diverse unnatural polyketides which is now in progress in our laboratories.

3. Experimental

3.1. Chemicals

p-Coumaroyl-CoA was chemically synthesized according to the published method (Stöckigt and Zenk, 1975). Malonyl-CoA, butyryl-CoA, hexanoyl-CoA, octanoyl-CoA, decanoyl-CoA, dodecanoyl-CoA, tetradecanoyl-CoA, hexadecanoyl-CoA, and octadecanoyl-CoA were purchased from Sigma. [2-^{14}C]Malonyl-CoA (48 mCi/mmol) was purchased from Moravek Biochemicals (California). 4-Hydroxy-6-pentyl-2-pyrone (**8a**) and 4-hydroxy-6-(2-oxoheptyl)-2-pyrone (**9a**) were obtained in our previous works (Morita et al., 2000, 2001).

3.2. Enzymes

Recombinant *S. baicalensis* CHS and *A. hypogaea* STS with an additional hexahistidine tag at the C-terminal were expressed in *Escherichia coli*, and purified by Ni-chelate affinity chromatography as described before (Abe et al., 2000, 2001, 2002, 2003a,b; Morita et al., 2000, 2001). Protein concentration was determined by the Bradford method (Protein Assay, Bio-Rad) with bovine serum albumin as standard.

3.3. Enzyme reaction

The standard reaction mixture contained 27 nmol of CoA ester of long-chain fatty acid (C_4–C_{18}), 54 nmol of malonyl-CoA, and 5 μg of the purified enzyme in a final volume of 500 μL of 100 mM potassium phosphate buffer, pH 8.0, containing 1 mM EDTA. Incubations were carried out at 30 °C for 20 min to overnight, and stopped by adding 50 μL of 20% HCl. The products were then extracted with 1 mL of ethyl acetate (×2), and concentrated by N_2 flow. The residue was dissolved in aliquot of MeOH containing 0.1% TFA, and separated by HPLC as described below.

3.4. Enzyme kinetics

Steady-state kinetic parameters were determined by using [2-^{14}C]malonyl-CoA (1.8 mCi/mmol) as a substrate. The experiments were carried out in triplicate

using five concentrations of long-chain fatty acid (C_6, C_8, C_{10}, and C_{12}) in the assay mixture, containing 27 μM of malonyl-CoA, 5 μg of purified enzyme, 1 mM EDTA, in a final volume of 500 μL of 100 mM Tris–HCl buffer, pH 8.0. Incubations were carried out at 30 °C for 20 min. The reaction products were extracted and separated by TLC (Merck Art. 1.11798 Silica gel 60 F_{254}; ethyl acetate/hexane/AcOH = 63:27:5, v/v/v). Radioactivities were quantified by autoradiography using a bioimaging analyzer BAS-2000II (FUJIFILM). Lineweaver–Burk plots of data were employed to derive the apparent K_M and k_{cat} values (average of triplicates ± standard deviation) using EnzFitter software (BIOSOFT).

3.5. HPLC and HPLC-ESIMS

The enzyme reaction products were separated by reverse phase HPLC (JASCO 880, JASCO) on TSK-gel ODS-80Ts column (4.6 x 150 mm, TOSOH) with a flow rate of 0.8 mL/min. Elutions were monitored by a multichannel UV detector (MULTI 340, JASCO) at 290, 330 and 360 nm; UV spectra (198–400 nm) were recorded every 0.4 s. Gradient elution was performed with H_2O (A) and MeOH (B) both containing 0.1% TFA. The gradient profile 1 for the reaction products of hexanoyl-CoA (**7a**): 0–5 min, A:B (7:3); 5–17 min, A:B (7:3 → 4:6); 17–25 min, A:B (4:6); 25–27 min, A:B (4:6 → 3:7). Profile 2 for the reaction products of octanoyl-CoA (**7b**): 0–5 min, A:B (4:6); 5–7 min, A:B (4:6 → 3:7); 7–15 min, A:B (3:7); 15–17 min, A:B (3:7 → 2:8); 17–25 min, A:B (2:8); 25–27 min, A:B (2:8 → 1:9); 27–35 min, A:B (1:9); 35–37 min, A:B (1:9 → 0:100); 37–45 min, B(100%). Profile 3 for the reaction products of decanoyl-CoA (**7c**): 0–5 min, A:B (3:7); 5–7 min, A:B (3:7–2:8); 7–15 min, A:B (2:8); 15–17 min, A:B (2:8 → 1:9); 17–25 min, A:B (1:9); 25–27 min, A:B (1:9 → 0:100); 27–45 min, 100% B. Profile 4 for the reaction products of dodecanoyl-CoA (**7d**): 0–5 min, A:B (2:8); 5–7 min, A:B (2:8 → 1:9); 7–15 min, A:B (1:9); 15–17 min, A:B (1:9 → 0:100); 17–45 min, 100% B. Profile 5 for the reaction products of myristoyl-CoA (**7e**), palmitoyl-CoA (**7f**), and stearoyl-CoA (**7g**): 0–5 min, A:B (7:3); 5–17 min, A:B (7:3 → 4:6); 17–25 min, A:B (4:6); 25–27 min, A:B (4:6 → 3:7); 27–35 min, A:B (3:7); 35–37 min, A:B (3:7 → 1:3); 37–45 min, A:B (1:3); 45–47 min, A:B (1:3 → 1:4); 47–55 min, A:B (1:4); 55–57 min, A:B (1:4 → 1:9); 57–65 min, A:B (1:9); 65–67 min, A:B (1:9 → 5:95); 67–75 min, A:B (5:95); 75–77 min, A:B (5:95 → 0:100); 77–85 min, 100% B.

On-line HPLC-ESIMS spectra were measured with a Hewlett–Packard HPLC 1100 series (Wilmington, DE) coupled to a Finnigan MAT LCQ ion trap mass spectrometer (San Jose, CA) fitted with an ESI source. HPLC separations were carried out under the same conditions as described above. The ESI capillary temperature and capillary voltage were 225 °C and 3.0 V, respectively. The tube lens offset was set at 20.0 V. All spectra were obtained in the negative and positive mode; over a mass range of *m/z* 100–500, at a range of one scan every 2 s. The collision gas was helium, and the relative collision energy scale was set at 30.0% (1.5 eV).

3.6. Spectroscopic data for the enzyme reaction products

Products from hexanoyl-CoA (**7a**). Compound **8a**: HPLC: R_t = 31.4 min. LC-ESIMS: MS, *m/z* 183 $[M + H]^+$, 181 $[M - H]^-$, MS/MS (precursor ion at *m/z* 181), *m/z* 137 $[M - H–CO_2]^-$. UV: λ_{max} 285 nm. Compound **9a**: HPLC: R_t = 26.3 min. LC-ESIMS: MS, *m/z* 225 $[M + H]^+$, 223 $[M - H]^-$, MS/MS (precursor ion at *m/z* 223), *m/z* 179 $[M - H–CO_2]^-$, *m/z* 125 $[C_6H_5O_3]^-$. UV: λ_{max} 291 nm. Products from octanoyl-CoA (**7b**). Compound **8b**: HPLC: R_t = 21.6 min. LC-ESIMS: MS, *m/z* 211 $[M + H]^+$, 209 $[M - H]^-$, MS/MS (precursor ion at *m/z* 209), *m/z* 165 $[M - H–CO_2]^-$. UV: λ_{max} 285 nm. Compound **9b**: HPLC: R_t = 16.1 min. LC-ESIMS: MS, *m/z* 253 $[M + H]^+$, 251 $[M - H]^-$, MS/MS (precursor ion at *m/z* 251), *m/z* 207 $[M - H–CO_2]^-$, *m/z* 125 $[C_6H_5O_3]^-$. UV: λ_{max} 291 nm. Products from decanoyl-CoA (**7c**). Compound **8c**: HPLC: R_t = 21.7 min. LC-ESIMS: MS, *m/z* 239 $[M + H]^+$, 237 $[M - H]^-$, MS/MS (precursor ion at *m/z* 237), *m/z* 193 $[M - H–CO_2]^-$. UV: λ_{max} 285 nm. Products from dodecanoyl-CoA (**7d**). Compound **8d**: HPLC: R_t = 17.7 min. LC-ESIMS: MS, *m/z* 267 $[M + H]^+$, 265 $[M - H]^-$, MS/MS (precursor ion at *m/z* 265), *m/z* 221 $[M - H–CO_2]^-$. UV: λ_{max} 285 nm. Products from tetradecanoyl-CoA (**7e**). Compound **8e**: HPLC: R_t = 75.6 min. LC-ESIMS: MS, *m/z* 295 $[M + H]^+$, 293 $[M - H]^-$ MS/MS (precursor ion at *m/z* 293), *m/z* 249 $[M - HCO_2]^-$. UV: λ_{max} 285 nm.

Note added in proof

Recently solved crystal structure of a bacterial type III PKS revealed a novel cavity extending into the floor of the active site of the enzyme, providing explanation for its extra polyketide extension activities when primed with CoA esters of long-chain fatty acid. (Austin, M. B., Izumikawa, M., Bowman, M. E., Udwary, D. W., Ferrer, J.-L., Moore, B., Noel, J.P., 2004. Crystal structure of a bacterial type III polyketide synthase and enzymatic control of reactive polyketide intermediate. J. Biol. Chem., published on WEB on August 19.)

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