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Note

Characterization of 2-Octenoyl-CoA Carboxylase/Reductase Utilizing *pteB* from *Streptomyce avermitilis*

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The filipin biosynthetic gene cluster of *Streptomyces avermitilis* contains *pteB*, a homolog of crotonyl-CoA carboxylase/reductase. PteB was predicted to be 2-octenoyl-CoA carboxylase/reductase, supplying hexylmalonyl-CoA to filipin biosynthesis. Recombinant PteB displayed selective reductase activity toward 2-octenoyl-CoA while generating a broad range of alkylmalonyl-CoAs in the presence of bicarbonate.

Key words: 2-octenoyl-CoA carboxylase/reductase; crotonyl-CoA carboxylase/reductase; *Streptomyces avermitilis*; polyketide synthase; filipin biosynthesis

Crotonyl-CoA reductase (CCR) of *Rhodobacter sphaeroides* was recently redefined as crotonyl-CoA carboxylase/reductase, supplying ethylmalonyl-CoA in C_5-dicarboxylic acid biosynthesis.[1,2] It was immediately recognized that CCR might play a role in supplying C_4-extension unit, in the form of ethylmalonyl-CoA, in polyketide biosynthesis.[1] Many bacterial polyketide biosynthetic gene clusters harbor a homolog of *ccr*, and the cognate polyketide structure incorporates the C_4-extension unit. This is exemplified by the FK520 biosynthetic gene cluster.[3] In the catalysis of modular polyketide synthase (PKS), an acyltransferase domain (AT) selects a malonyl-CoA or an alkylmalonyl-CoA precursor and charges the neighboring acyl-carrier protein (ACP) with it. β-Ketoacyl-ACP synthase incorporates the selected precursor unit into the growing polyketide chain through decarboxylative Claisen condensation. The precursor selection step is pivotal in generating the structural diversity of polyketides. Extensive research efforts have hence been directed toward understanding the biosynthesis of atypical malonyl-CoA derivatives and the biochemical mechanisms guiding their selective incorporation into growing polyketide chains.[4]

Biochemical characterization of the CCR route in polyketide biosynthesis was first reported for the salinosporamide biosynthesis of *Salinospora tropica*.[5,6] SalG catalyzes the formation of ethylmalonyl-CoA, propylmalonyl-CoA, and chloroethylmalonyl-CoA to support the production of salinosporamide derivatives. The metabolic significance of SalG might lie in its production of chloroethylmalonyl-CoA. Strop_3612 encodes a CCR with housekeeping properties that might act as the major biosynthetic machinery in producing ethylmalonyl-CoA in *S. tropica*.

The polyketide biosynthetic gene cluster of filipin in *Streptomyces avermilitis* contains a homolog of the CCR gene, *pteB*.[7,8] The chemical structure of filipin suggests that the last chain-extension step incorporates hexylmalonyl-CoA. This is the basis of our hypothesis that PteB is 2-octenoyl-CoA carboxylase/reductase (Fig. 1AB). Notably, the *S. avermitilis* genome also harbors at least two other CCR encoding genes (one previously identified in the oligomycin biosynthetic gene cluster).[7] We found that recombinant PteB selectively reduced 2-octenonyl-CoA in the absence of bicarbonate while inclusion of bicarbonate drove PteB to catalyze reductive carboxylation toward crotonyl-, 2-hexenoyl-, 2-octenoyl-, and 2-decenoyl-CoA.

Polymerase chain reaction (PCR) was used to amplify *pteB* from the total DNA of *S. avermitilis* DNA utilizing the following primers: 5′-ATTCATATGACGAAAGCTCTCTACGA-3′ and 5′-TTCAAGCTTTCAGGCGGCGGCGAGTTCGA-3′ (engineered *Nde*I and *Hind*III sites underlined). PCR-amplified *pteB* was sub-cloned in pET28a, and protein synthesis was induced in *E. coli* BL21 (*DE3*) with 1 mM isopropyl β-D-1-thiogalactopyranoside at 14 °C overnight (Fig. 1C). 2-Alkenoyl-CoA substrates, except for crotonyl-CoA, were generated by reaction of the cognate alkanoyl-CoA with acyl-CoA oxidase (ACOD, Wako Pure Chemical Industries, Osaka, Japan). Crotonyl-CoA, hexanoyl-CoA, octanoyl-CoA, and decanoyl-CoA were purchased from Sigma-Aldrich (St. Louis, MO). One mM alkanoyl-CoA was incubated with 0.1 U ACOD in 100 µL of 100 mM potassium bicarbonate and 50 mM Tris–HCl (pH 7.5) for 20 min at 37 °C. The reaction mixture was boiled for 2 min, and transferred to an ice bath, and then 2 mM reduced nicotinamide adenine dinucleotide phosphate (NADPH) and 3 µM PteB were added. The final concentration of potassium bicarbonate was 80 mM in the PteB reaction. Crotonyl-CoA was used in the reaction without ACOD treatment. High-performance liquid chromatography (HPLC) indicated that ACOD successfully generated 2-alkenyl-CoA, as verified by negative ESI-MS analysis utilizing the detection of

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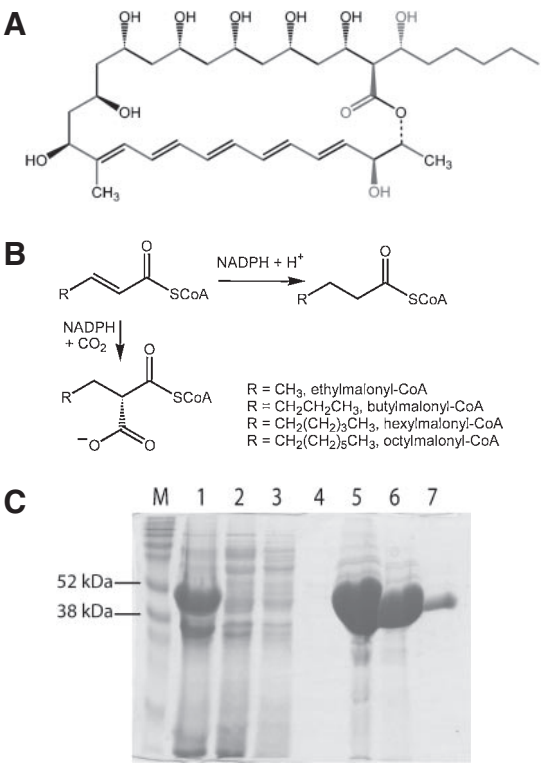

Fig. 1. Filipin III Structure (A), Reaction Scheme for 2-Alkenoyl-CoA Carboxylase/Reductase (B), and Purification of PteB (C).

C, Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of 6 × histidine tagged recombinant PteB purified by nickel affinity chromatography on Ni-NTA agarose. Lane M, molecular weight protein markers (Amersham; 225, 150, 102, 76, 52, 38, 31, 24, 17, and 12 kDa top to bottom respectively with 52- and 38-kDa proteins indicated); lane 1, total proteins; lane 2, flow-through; lane 3, elution with 20 mM imidazole; lanes 4–7, elution with 250 mM imidazole.

$[M - H^+]^-$ at m/z 862.3, 890.3, and 918.2 for 2-hexenoyl-, 2-octenoyl-, and 2-decenoyl-CoA respectively (Fig. 2). Alkanoyl-CoA substrate was left in the various ACOD reactions, and complete conversion to 2-alkenoyl-CoA was not achieved because the ACOD reaction generates by-products when incubated for longer than 20 min.

The various PteB reactions generated new peaks with consumption of 2-alkenoyl-CoAs (Fig. 2). The samples corresponding to the new peaks were collected and analyzed by mass spectrometry. Electrospray ionization-mass spectrometry (ESI-MS) in negative mode verified the identities of the alkylmalonyl-CoAs by accessing the detection of $[M - 2H^+]^{2-}$ at m/z 439.7, 453.6, 467.7, and 481.6 for ethyl, butyl, hexyl, and octylmalonyl-CoA respectively. The conversion of crotonyl-CoA into ethylmalonyl-CoA was not completed, and ESI-MS verified the identity of the crotonyl-CoA that was left in the PteB reaction (Fig. 2A). Analyses of these HPLC results indicated that recombinant PteB displays broad substrate specificity for *in vitro* reductive carboxylation. Notably, the level of alkanoyl-CoA was slightly enhanced in the PteB reaction, especially with 2-decenoyl-CoA. This enhanced level indicates that some 2-alkenyl-CoAs are converted into the cognate alkanoyl-CoAs through reduction decoupled with carboxylation (Fig. 1B). When the bicarbonate concentration was reduced to 1 mM, the carboxylation-decoupled reduction appeared enhanced, whereas overall reductive conversion was diminished (data not shown).

The substrate specificity of PteB was further examined in a UV-Vis spectrophotometer by monitoring of NADPH oxidation. This experiment showed that PteB selectively reduced 2-octenoyl-CoA in the absence of bicarbonate (Fig. 3A). Marginal NADPH oxidation was observed with 2-hexenoyl-CoA. When 80 mM bicarbonate was included, PteB displayed broad substrate specificity,

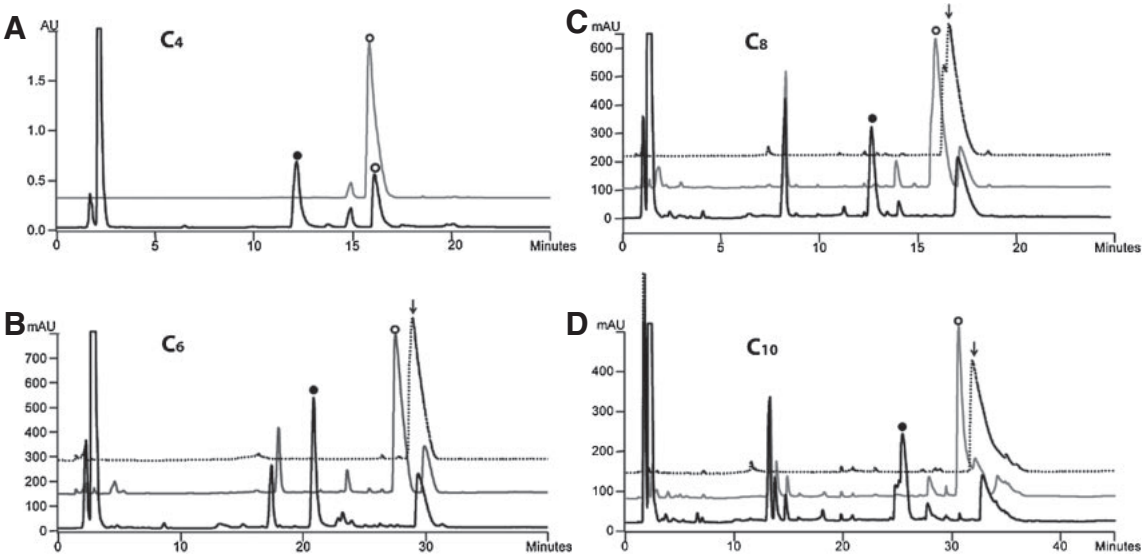

Fig. 2. HPLC Analysis of PteB Reactions with 2-Alkenoyl-CoAs.

PteB reaction with crotonyl-CoA (A) and ACOD reactions (B–D) are shown by black solid lines; hexanoyl-CoA (B), octanoyl-CoA (C), and decanoyl-CoA (D). ACOD reaction samples and authentic alkanoyl-CoAs are shown by gray solid lines and black dotted lines respectively. Alkylmalonyl-CoAs, 2-alkenoyl-CoAs, and alkanoyl-CoAs are indicated by solid circles, hollow circles, and arrows respectively. The reactions were monitored by HPLC on a Gemini C-18 column (150 × 2 mm, 3.0 μm; Phenomenex, Torrance, CA) at 260 nm. The mobile phase consisted of 5 mM ammonium acetate and 5% methanol in water (A) and methanol (B). The flow rate was maintained at 0.5 mL/min. The column was programmed to run in a timed gradient fashion: 100% A for 5 min, transitioning from 100% A to 10% A over the next 25 min, then maintained at 10% A for 10 min. The sample injection volume was 25 μL.

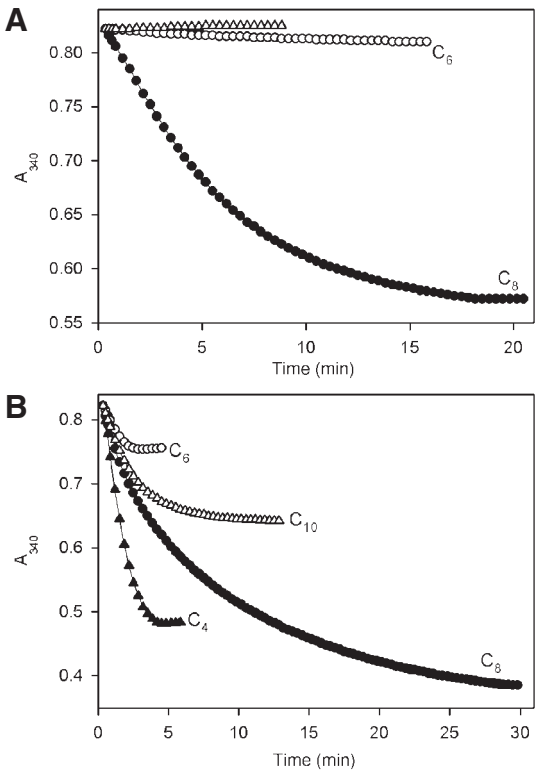

Fig. 3. Spectrophotometric Analysis of PteB Reactions with 2-Alkenoyl-CoAs.

PteB reactions with crotonyl-CoA (solid triangles), ACOD reaction of hexanoyl-CoA (hollow circles), octanoyl-CoA (solid circles), and decanoyl-CoA (hollow triangles) were performed in 50 mM Tris–HCl (pH 7.5) using BRAND UV-cuvette micro (BrandTech Scientific, Wertheim, Germany) without potassium bicarbonate (A) and with 80 mM potassium bicarbonate (B). NADPH consumption in the 100-μL reaction volume was monitored at 340 nm at ambient temperature. The absorbance values of the experiments were adjusted to be identical at the initial time points.

as seen in the HPLC analysis (Fig. 3B). The apparent kinetic properties of PteB were found to be comparable for 2-octenoyl-CoA and crotonyl-CoA (data not shown). These results indicate that PteB is 2-octenoyl-CoA reductase with properties consistent with relaxed substrate specificity in reductive carboxylation. SalG also possesses relaxed substrate specificity toward crotonyl-CoA, 2-pentenyl-CoA, and 4-chlorocrotonyl-CoA.[5,6)]

2-Pentenoyl-CoA carboxylase/reductase (AllR) was proposed to play a part in FK 506 biosynthesis.[9)] TcsC (a synonym for AllR) was recently characterized as 2-pentenoyl-ACP carboxylase/reductase, in that it possessed a low level of 2-pentenoyl-CoA carboxylase/reductase activity.[10)] In *Streptomyces tsukubaensis*, FK506 polyketide synthase (PKS) produces FK506 and FK520 by utilizing allymalonyl-thioetser and ethylmalonyl-thioetser respectively. This indicates that the cognate AT of FK506 PKS has relaxed substrate specificity, as seen for salinosporamide biosynthesis.[4,5)]

It has been reported that a thuggacin biosynthetic gene cluster of myxobacteria *Sorangium cellulosun* contains 2-octenoyl-CoA carboxylase/reductase (TgaD).[11)] Phylogenetic analysis indicated that TugD and PteB constitute separate clades (data not shown). It is likely that *tugD* and *pteB* evolved independently in myxobacteria and actinobacteria respectively. All known thuggacins of *S. cellulosun* origin possess a hexyl group at the α-position of the macrolactone ring. An identical substituent is also present in filipins. Thus it can be hypothesized that the cognate AT of filipin and thuggacin PKS selectively incorporate hexylmalonyl-CoA. It is speculated that PteB retains relaxed substrate specificity due to *pteB* evolution from the *ccr* gene.

In conclusion, 2-octenoxyl-CoA carboxylase/reductase was characterized in filipin biosynthesis. PteB can be employed in combinatorial biosynthesis to generate polyketide structures from various alkyl precursors if the biosynthetic reaction involves an AT with relaxed substrate specificity, as seen for salinosporamide and FK506.[6,9)]

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References

1) Erb TJ, Berg IA, Brecht V, Müller M, Fuchs G, and Alber BE, *Proc. Natl. Acad. Sci. USA*, **104**, 10631–10633 (2007).
2) Erb TJ, Brecht V, Fuchs G, Müller M, and Alber BE, *Proc. Natl. Acad. Sci. USA*, **106**, 8871–8876 (2009).
3) Wu K, Chung L, Revill WP, Katz L, and Reeves CD, *Gene*, **251**, 81–89 (2000).
4) Chan YA, Podevels AM, Kevany BM, and Thomas MG, *Nat. Prod. Rep.*, **26**, 90–114 (2009).
5) Eustáquio AS, McGlinchey RP, Liu Y, Hazzard C, Beer LL, Florova G, Alhamadsheh MM, Lechner A, Kale AJ, Kobayashi Y, Reynolds KA, and Moore BS, *Proc. Natl. Acad. Sci. USA*, **106**, 11295–11300 (2009).
6) Liu Y, Hazzard C, Eustáquio AS, Reynolds KA, and Moore BS, *J. Am. Chem. Soc.*, **131**, 10376–10377 (2009).
7) Omura S, Ikeda H, Ishikawa J, Hanamoto A, Takahashi C, Shinose M, Takahashi Y, Horikawa H, Nakazawa H, Osonoe T, Kikuchi H, Shiba T, Sakaki Y, and Hattori M, *Proc. Natl. Acad. Sci. USA*, **98**, 12215–12220 (2001).
8) Xu LH, Fushinobu S, Takamatsu S, Wakagi T, Ikeda H, and Shoun H, *J. Biol. Chem.*, **285**, 16844–16853 (2010).
9) Goranovic D, Kosec G, Mrak P, Fujs S, Horvat J, Kuscer E, Kopitar G, and Petkovic H, *J. Biol. Chem.*, **285**, 14292–14300 (2010).
10) Mo S, Kim DH, Lee JH, Park JW, Basnet DB, Ban YH, Yoo YJ, Chen SW, Park SR, Choi EA, Kim E, Jin YY, Lee SK, Park JY, Liu Y, Lee MO, Lee KS, Kim SJ, Kim D, Park BC, Lee SG, Kwon HJ, Suh JW, Moore BS, Lim SK, and Yoon YJ, *J. Am. Chem. Soc.*, **133**, 976–986 (2011).
11) Buntin K, Irschik H, Weissman KJ, Luxenburger E, Blöcker H, and Müller R, *Chem. Biol.*, **17**, 342–356 (2010).