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Roles of Conserved Active Site Residues in the Ketosynthase Domain of an Assembly Line Polyketide Synthase

Thomas Robbins[†], Joshua Kapilivsky[†], David E. Cane[‡], and Chaitan Khosla^{*,†}

[†]Departments of Chemistry and Chemical Engineering, Stanford University, Stanford, California 94305, United States

[‡]Department of Chemistry, Brown University, Providence, Rhode Island 02912-9108, United States

Abstract

Ketosynthase (KS) domains of assembly line polyketide synthases (PKSs) catalyze intermodular translocation of the growing polyketide chain as well as chain elongation via decarboxylative Claisen condensation. The mechanistic roles of ten conserved residues in the KS domain of Module 1 of the 6-deoxyerythronolide B synthase were interrogated via site-directed mutagenesis and extensive biochemical analysis. Although the C211A mutant at the KS active site exhibited no turnover activity, it was still a competent methylmalonyl-ACP decarboxylase. The H346A mutant exhibited reduced rates of both chain translocation and chain elongation, with a greater effect on the latter half-reaction. H384 contributed to methylmalonyl-ACP decarboxylation, whereas K379 promoted C–C bond formation. S315 played a role in coupling decarboxylation to C–C bond formation. These findings support a mechanism for the translocation and elongation half-reactions that provides a well-defined starting point for further analysis of the key chain-building domain in assembly line PKSs.

Graphical abstract

Multimodular assembly line polyketide synthases (PKSs) are responsible for the biosynthesis of numerous medicinally important natural products.¹ These megasynthases

*Corresponding Author: khosla@stanford.edu. Telephone: (650) 723-6538.

Supporting Information

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Notes

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elongate polyketide chains via a series of decarboxylative Claisen condensation reactions in the active sites of their β -ketoacyl-ACP synthase (ketosynthase, KS) domains. Each KS resides within a catalytic unit termed a “module”, wherein the necessary enzymatic machinery for polyketide chain processing is also housed. In the case of the 6-deoxyerythronolide B synthase (DEBS), a prototypical assembly line PKS, the catalytic cycle of Module 1 (M1) is depicted in Figure 1A. The cycle begins with the translocation (Step I) of a propionyl primer unit from the upstream acyl carrier protein (ACP) of the loading didomain (LDD) onto the active site Cys residue of the KS. In Step II an extender unit is transacylated from (2*S*)-methylmalonyl-CoA onto the ACP via the acyltransferase (AT). The ACP is a critical domain of each module, as it shuttles intermediates within and between modules via a phosphopantetheine (Ppant) post-translational modification. In Step III the electrophilic KS-bound propionyl thioester undergoes decarboxylative Claisen condensation with the ACP-bound methylmalonyl group, resulting in elongation of the polyketide backbone by two carbon units. The stereochemistry and oxidative state of the β carbon atom of the resulting diketide, as well as the stereochemistry of its α carbon atom, is set by the ketoreductase (KR) domain of Module 1. Following condensation, although the active site of the KS is no longer occupied, it is unable to accept another propionyl unit from the LDD until the fully processed diketide intermediate has been translocated to the KS domain of Module 2 (Step IV). This hallmark of an assembly line PKS precludes premature entry of a growing polyketide chain into an acceptor module, and has been referred to as a “turnstile”.²

Although KS domains of assembly line PKSs have not yet been subjected to detailed mechanistic investigation, the enzymology of their homologues from bacterial and mammalian fatty acid synthases has been extensively studied over the past few decades.^{3–5} Acylation of the active site Cys of a fatty acid KS is believed to be mediated by the dipole moment of an α -helix located immediately behind the thiol⁶ (Figure 1B) as well as an oxyanion hole formed by the amide hydrogens of the active site Cys and a conserved Phe,⁵ whereas decarboxylative Claisen condensation is mediated by two conserved His residues whose precise roles have not been definitively established. Both these features are likely to be conserved in KS domains of assembly line PKSs. In contrast, while the KS of a fatty acid synthase interacts with the same ACP through the entire biosynthetic cycle, its counterparts in assembly line PKSs must interact with different ACP domains during intermodular chain translocation (Step I; Figure 1A) versus intramodular chain elongation (Step III; Figure 1A). Moreover, fatty acid synthases do not require a turnstile mechanism, because the growing fatty acid chain is passed iteratively between the same pair of KS and ACP thiols.

By comparing the amino acid sequences of two subgroups of KS domains—those from assembly line PKSs and those from iterative PKSs—we generated a 90% consensus sequence for each subgroup, the conserved residues for which are depicted in red on the noniterative DEBS KS1 and the iterative Lovastatin B KS (Figure 2). Alignment of these two consensus sequences led to the identification of residues within 10 Å of the active site Cys (as determined from the crystal structures of KS domains of assembly line PKSs^{7,8}) that were either universally conserved (C211, H346, K379, and H384) or only conserved within each subgroup but different between the two (T209, S315, N381, Q386, N455, H457). Each of these residues in the KS domain of DEBS Module 1 was selected for mutation. In most

cases the wild-type residue was replaced with Ala. In the case of N455L, however, an isosteric change was introduced, whereas for Q386E the corresponding residue found in the subgroup of iterative KSs was introduced. Residue numbering corresponds to the KS domain of Module 1, starting from the N-terminus of a previously described engineered construct.⁹ To our knowledge, this is the first systematic mutational analysis of a ketosynthase domain from an assembly line PKS. Therefore, not only do these findings improve our understanding of the universally conserved mechanistic features of this signature component of PKSs, but they also shed new light on the principles of vectorial biosynthesis by enzymatic assembly lines.

MATERIALS AND METHODS

Materials—Reagents and Chemicals

In-Fusion HD Cloning Kit was from Clontech. Phusion High Fidelity polymerase was from Thermo Scientific. All primers for Quickchange mutagenesis were synthesized by Elim Biopharmaceuticals, and those primers used for In-Fusion Cloning were from Pan-Oligo. Mass spectrometry grade Trypsin/Lys-C protease was from Promega Corporation. Primers used to generate mutants are listed in Table S1.

Bacterial Cell Culture and Protein Purification

All chemicals for preparation of buffers were from Sigma-Aldrich. Isopropyl- β -D-1-thiogalactopyranoside (IPTG) was from Gold Biotechnology. Luria–Bertani (LB) Miller Broth was from Fisher Scientific. Halt protease inhibitor cocktail was from Thermo Scientific. Ni-NTA affinity resin was from MC Lab. HiTrap Q anion exchange chromatography columns for protein purification were from GE Healthcare. SDS-PAGE Mini Protean TGX Precast Gels were from BioRad, and Amicon Ultra centrifugal filters were from Millipore.

Enzymatic Assays

Coenzyme A (CoASH), reduced β -nicotinamide adenine dinucleotide 2'-phosphate (NADPH), methylmalonic acid, *N*-ethylmaleimide, butyryl-CoA, sodium propionate, sodium phosphate, and magnesium chloride hexahydrate were from Sigma-Aldrich. Adenosine-5'-triphosphate (ATP) was from Teknova. Bond-breaker tris(2-carboxyethyl)phosphine (TCEP) was from Thermo Scientific. UVette cuvettes (2 mm $\times$ 10 mm path) were from Eppendorf. Methanol, ethyl acetate, and HPLC grade acetonitrile were from Fisher Scientific. Urea was from J. T. Baker. Formic acid was from Fluka. [¹⁴C]-Propionate was from American Radiochemicals. Methylmalonyl-CoA was from Santa Cruz Biotechnology.

Plasmid Construction

Expression plasmids for all Module 1 mutants of DEBS (with the exception of K379A, which was constructed using an In-Fusion HD Cloning Kit) were constructed using the Quickchange site-directed mutagenesis with plasmid pBL13 as the template.⁹ The numbering of the mutants corresponds to the residue position in pBL13. The identity of each construct was confirmed by DNA sequencing.

Bacterial Cell Culture and Protein Purification

Wild-type Module 1 and each of its mutants—H346A, H384A, K379A, C211A, N455L, N381A, Q386E, H457A, T209A, and S315A—were expressed and purified using a similar protocol. Expression plasmids were introduced into *E. coli* BAP1 cells to allow phosphopantetheinyl modification of ACP domains.¹⁰ Overnight seed cultures were used to inoculate a 1 L culture containing carbenicillin. Cells were grown to an approximate O.D. of 0.6, and induced with 250 μ L of 1 M IPTG. After 16 h, cells were harvested by centrifugation at 4420 *g* for 10 min and lysed by sonication in lysis buffer (50 mM sodium phosphate, 10 mM imidazole, 450 mM NaCl, 20% glycerol, pH = 7.6). The lysate was clarified by centrifugation at 25 000 *g*, and the supernatant was added to Ni-NTA agarose resin (2 mL of resin per L of culture) and allowed to incubate at 4 °C for 1 h. The incubating resin was applied to a Kimble-Kontes Flex column and washed with 20 column volumes of lysis buffer, 10 column volumes of wash buffer (50 mM phosphate, 25 mM imidazole, 300 mM NaCl, 10% glycerol, pH = 7.6), and eluted with 4 column volumes of elution buffer I (75 mM phosphate, 150 mM imidazole, 40 mM NaCl, 10% glycerol, pH = 7.6) and 4 column volumes of elution buffer II (75 mM phosphate, 500 mM imidazole, 40 mM NaCl, 10% glycerol, pH = 7.6). The eluent was further purified using anion exchange chromatography (HiTrap Q column, Buffer A: 50 mM phosphate, pH = 7.6, 10% glycerol and Buffer B: 50 mM phosphate, 500 mM NaCl, pH = 7.6, 10% glycerol) on an AKTA Pure FPLC system. FPLC fractions were analyzed by SDS-PAGE, and fractions containing the desired protein were pooled and concentrated using an Amicon Ultra filter. Protein concentrations were measured using the Pierce BCA Protein Assay Kit (Thermo Scientific). Protein samples were aliquoted and stored at –80 °C. For H346A, H384A, and K379A mutants, a Thermo Scientific Halt protease inhibitor cocktail was added to the lysis buffer to improve protein yield. Protein yields ranged from 0.5 to 4.8 mg/L of cell culture. The LDD and Module 2+TE proteins were purified as previously described.⁹

The *S. coelicolor* MatB, methylmalonyl-CoA epimerase (SCME), and propionyl-CoA synthetase (PrpE) were also purified using the above protocol and their activity was verified by the UV-Spectrophotometric Kinetic Assay described below. See Figure S1 for SDS-PAGE gels (stained with SimplyBlue SafeStain from Invitrogen) and yields of all Module 1 proteins.

UV Spectrophotometric Kinetic Assays

Kinetic parameters (k_{cat} , K_{50}) were determined by fitting the data using GraphPad Prism 6 to the equation: $V/[E]_0 = k_{\text{cat}}[S]/(K_{50} + [S])$, where $V/[E]_0$ is the initial rate normalized to the concentration of proteins in the assay that were held constant and $[S]$ is the concentration of the LDD that is being varied. Parameters are reported as the fitting value $\pm$ s.e. ($n = 3$) with standard error derived from the nonlinear curve fitting. Error bars on graphs represent the mean $\pm$ s.d. ($n = 3$).

Assays to measure the initial rates of polyketide synthesis were performed as follows. Reactions were performed on a 65 μ L scale containing 400 mM sodium phosphate (pH = 7.2), 4% glycerol, 5 mM TCEP, 10 mM MgCl₂, 2 mM coenzyme A, and 2.5 mM ATP. Enzymes MatB (2 μ M) and methylmalonyl-CoA epimerase (4 μ M) were added to convert

methylmalonate into racemic methylmalonyl-CoA.¹¹ Enzyme PrpE (2 μ M) was added to convert propionate into propionyl-CoA.² The concentration of these three enzymes was selected to ensure that acyl-CoA supply was not rate limiting. To the above reaction mixture were added the Module 2 +TE (1 μ M), wild-type Module 1 or its mutants (1 μ M in all cases except for S315A and N381A, which were present at 1.1 and 1.2 μ M respectively), and LDD, whose concentration was varied between 0 and 4 μ M. To assay the upper bound of the turnover for the K379A mutant, the following concentrations of DEBS proteins were used (LDD = 2 μ M, K379A = 20 μ M, Module 2 +TE = 2 μ M). Reactions were initiated upon the addition of NADPH (500 μ M), propionate (1 mM), and methylmalonate (1 mM). The reaction was carefully mixed 20 times and 55 μ L were transferred to an Eppendorf UVette cuvette and monitored at 340 nm for the depletion of NADPH using a Lambda-25 UV-vis Spectrophotometer (PerkinElmer) for 10 min at room temperature.

Verification of Product Identity by LC/MS

Assays to verify the products of the wild-type and mutant bimodular PKSs were performed as follows. Each reaction vessel contained 400 mM sodium phosphate buffer (pH = 7.2), 4% glycerol, 5 mM TCEP, and LDD (2 μ M), wild-type or mutant Module 1 (2 μ M), and Module 2+TE (2 μ M) in a volume of 25 μ L. The K379A mutant of Module 1 was used at a final concentration of 20 μ M. To this protein mixture was added an equal volume of a substrate solution containing 400 mM sodium phosphate buffer (pH = 7.2), TCEP (5 mM), MgCl₂ (10 mM), coenzyme A (2 mM), ATP (3 mM), NADPH (1 mM), methylmalonate (1 mM), propionate (1 mM), and the following three enzymes: malonyl-CoA synthetase (MatB, 2 μ M), methylmalonyl-CoA epimerase (4 μ M), and propionyl-CoA synthetase (PrpE, 2 μ M). Reactions were incubated for 1 h, and quenched and extracted with 2 $\times$ 400 μ L ethyl acetate. Extracts were vacuum-dried and reconstituted in methanol. The samples were separated on a Gemini-NX C₁₈ column (Phenomenex, 5 μ m, 2 $\times$ 100 mm) connected to an Agilent 1260 HPLC over a 28 min linear gradient of acetonitrile from 3% to 95%, and then injected into a 6520 Accurate-Mass QTOF mass spectrometer.

Radiolabeling of Wild-Type and Mutant Module 1 Proteins

Assays to measure the occupancy of Module 1 were performed using a previously described radio-SDS-PAGE protocol.² One reaction vessel on a 25 μ L scale contained 400 mM sodium phosphate buffer (pH = 7.2), 4% glycerol, 5 mM TCEP, LDD (4 μ M), and wild-type or mutant Module 1 (4 μ M). An equal volume of the same substrate solution as above was added following preincubation for 1 h. Individual samples (10 μ L) were withdrawn at 1, 5, and 15 min and combined with an equal volume of Laemmli buffer lacking reducing agent. Samples were frozen rapidly, stored at -20 °C, and then thawed and analyzed on a 7.5% polyacrylamide gel (Bio-Rad) at 200 V for 45 min in an ice bath. The gels were washed with hot water for 7 min, stained with hot Simply Blue Safestain for 7 min, and destained for 1 min in water. Gels were transferred to a filter paper and dried in vacuo using a Bio-Rad 543 Gel dryer at 80 °C for 2 h. The extent of ¹⁴C-radiolabeling in each protein was quantified on a Rita Star TLC Analyzer (Raytest), and converted into a percent protein occupancy using a standard curve, as described previously.²

Acyl Carrier Protein Occupancy Assay

To assess the relative abundance of different acyl chains attached to the ACP domains of wild-type or mutant Module 1 proteins, the following assay was performed. A 15 μL mixture containing sodium phosphate buffer (pH = 7.2, 400 mM), 4% glycerol, TCEP (5 mM), LDD (4 μM), and wild-type or mutant Module 1 (4 μM) was mixed with a substrate mixture comprising NADPH (500 μM), methylmalonyl-CoA (500 μM), and butyryl-CoA (500 μM). At 15 min, the reaction was quenched with an equal volume of 8 M urea for 30 min. *N*-Ethylmaleimide (15 mM) was added to the resulting solution for 30 min in order to alkylate free thiols. The mixture was diluted with 5 vol equiv of sodium phosphate buffer (pH = 7.2, 200 mM, 10% glycerol), and 2 μL trypsin + LysC (1 mg/mL each) was added to proteolyze the denatured proteins for 90 min at 30 °C. The proteolysis reaction was quenched with the addition of formic acid (final concentration 5% w/v), and the resulting peptides were purified using a STAGE-tip procedure,¹² eluted with 80% acetonitrile in 5% formic acid, lyophilized, and reconstituted in 100 μL of 5% formic acid. Samples were separated on a Synergi 4u Hydro RP column (Phenomenex, 80 Å, 2 $\times$ 30 mm) connected to a Shimadzu 20 HPLC over a 3 min linear gradient of acetonitrile from 10% to 90%, and then injected into a Sciex Q-Trap 4000. The first quadrupole detected the trypsinized ACP fragment with appropriate modification, whereas the third quadrupole yielded the Ppant ejection product.¹³ The following potentials were used: Declustering Potential = 20 V, Entrance Potential = 20 V, Collision Energy = 77 V, and Collision Cell Exit Potential = 16 V. Given the limitations of the cycling speed of the instrument, Q-TRAP analysis was performed first on species **2**, **3**, and **4** with a 100 ms cycling, and then on species **2**, **3**, **5**, and **6**. To compare between runs, the ion counts of each species were normalized to the amount of species **2** observed.

RESULTS

Identification of Module 1 Mutants Deficient in Chain Translocation or Chain Elongation

As outlined above, ten mutants of the KS domain of Module 1 (C211A, H346A, K379A, H384A, T209A, S315A, N381A, Q386E, N455L, and H457A) were constructed, individually purified, and subjected to biochemical analysis. Initially we sought to establish whether each mutant was defective in translocation of the propionyl primer unit from the ACP domain of the LDD (Step I, Figure 1A) and/or elongation of the propionyl group with a methylmalonyl extender unit (Step III, Figure 1A). To discriminate between these possibilities, the steady state turnover rate of the bimodular PKS comprised DEBS LDD, Module 1, and Module 2+TE (Figure 3) was measured as described previously⁹ in the presence of varying concentrations of the LDD protein. Because DEBS Module 1 can self-prime via a decarboxylative priming mechanism in the absence of LDD,¹⁴ the reaction rate observed in its absence was subtracted from all other rate measurements prior to fitting the data to the Michaelis–Menten equation. Table 1 summarizes the k_{cat} , K_{50} , and k_{cat}/K_{50} values for the production of **1** in the presence of wild-type Module 1 and each of its ten mutants (where K_{50} refers to the concentration of LDD protein at which the observed turnover is half of its maximum value). Under these assay conditions, translocation of the propionyl primer from the ACP domain of the LDD onto the KS active site of Module 1 (Step I, Figure 1A) was expected to be the first irreversible step,¹⁵ implying that a change in

k_{cat}/K_{50} of a given mutant would reflect the role of the corresponding residue in this step of the catalytic cycle.

Three mutants of Module 1 (C211A, H346A, and K379A) had negligible product-forming activity under steady state turnover conditions. Upon closer investigation of the product profiles by mass spectrometry (Figure 4), the H346A mutant did produce detectable quantities of **1**, albeit only at ~1% the level of its wild-type counterpart. Activity of the K379A mutant could only be detected when the corresponding protein was added at a 10-fold higher concentration; even at such a high protein concentration, only ~1% of the wild-type activity could be observed spectrophotometrically. The active site Cys mutant of Module 1 (C211A) did not display detectable activity under any assay conditions. The k_{cat}/K_{50} values of two mutants, H384A and Q386E, were markedly lower than those for wild-type Module 1, whereas the corresponding parameter was moderately higher in the cases of the N381A and T209A mutants.

Single Turnover Analysis of Intermodular Chain Translocation

Recently we reported a radio-SDS-PAGE assay that not only allows quantitation of the transacylation rate from a donor ACP to an acceptor module, but also detects tight coupling between intermodular chain translocation and intramodular chain elongation.² When Module 1 of DEBS was coincubated with the LDD in the presence of ¹⁴C-propionyl-CoA, the rate at which Module 1 protein was labeled reflected the translocation rate of the propionyl primer from the ACP domain of the LDD to the KS domain of Module 1. If unlabeled methylmalonyl-CoA was also present in the reaction mixture, the propionyl primer also underwent elongation, and the radiolabel was transferred to the ACP domain of Module 1. However, due to the tight coupling between chain elongation and translocation, the KS domain of Module 1 did not react further and remained unoccupied as long as the diketide was anchored onto its intramodular partner ACP. Here, we sought to use this single-turnover assay to quantify the translocation efficiency of a propionyl primer unit from the ACP domain of LDD to the KS domain of each Module 1 mutant. The same assay was also expected to reveal if the strict energetic coupling between elongation and translocation observed in wild-type Module 1, corresponding to the turnstile mechanism, might be relaxed in any mutant.

As expected, the C211A mutant of Module 1 remained unlabeled at all time-points evaluated (1 min, 5 min, 15 min; data not shown), ruling out the possibility of nonspecific labeling of the ACP domain of Module 1 by the LDD under the assay conditions. As shown in Figure 5, the H384A and Q386E mutants of Module 1 had comparable chain translocation efficiency to the wild-type protein. Indeed, even the highly attenuated H346A and K379A mutants showed significant labeling. Given the marked effects of these residues on the steady state turnover assay outlined above (Figure 3 and Table 1), the data shown in Figure 5 suggested that all four residues had a considerably greater influence on chain elongation as compared to chain translocation. Interestingly, not only did the N455L mutant undergo rapid labeling, but it also showed higher steady state occupancy than the wild-type module. The remaining mutants interrogated by this assay did not differ appreciably from wild-type Module 1 (data not shown).

Competition between Translocation and Decarboxylative Self-Priming as a Source of Electrophilic Substrates of Module 1

In the steady state turnover assay shown in Figure 3, the propionyl thioester substrate of Module 1 can in principle be derived either from propionyl-CoA via translocation from the LDD or from methylmalonyl-CoA through Module 1-catalyzed decarboxylative self-priming. Discriminating between these two alternative sources of electrophilic primers is not straightforward. We therefore used butyryl-CoA as the source of primer units, since previous work had revealed that the LDD as well as Module 1 of DEBS can efficiently accept and process this unnatural substrate homologue.¹⁴ Under these conditions, it was anticipated that the mass of butyryl-derived ACP adducts could be readily differentiated by mass spectrometry from those of methylmalonyl-derived adducts. As detailed in the Materials and Methods section and illustrated in Scheme 1, DEBS LDD and Module 1 were coincubated with butyryl-CoA, methylmalonyl-CoA, and NADPH. Following a 15 min incubation, the MS-MS Ppant ejection method was used to quantify the relative abundance of the following species attached via a thioester linkage to the Ppant arm: N-ethylmaleimide (**2**, corresponding to an unoccupied module); methylmalonate (**3**, corresponding to AT-catalyzed transacylation of an extender unit); propionate (**4**, corresponding to KS-promoted decarboxylation of a methylmalonyl extender unit); propionyl-derived diketide (**5**, corresponding to elongation of a primer unit derived via decarboxylative self-priming); and butyryl-derived diketide (**6**, corresponding to the full catalytic cycle of Module 1). As expected, after incubation the ACP domain of wild-type Module 1 was present predominantly as species **5** or **6**, whereas some of the mutants presented interesting differences (Table 2).

The ACP domain of the C211A mutant gave rise to species **4** with an attached propionyl residue as its predominant Ppant ejection product, implying that the decarboxylative activity of this mutant was largely intact. The ACP of H346A revealed a mixture of all of the above species, with species **2** with an attached N-ethylmaleiamide being the most abundant, indicating that the terminal thiol of the original ACP domain had not been catalytically altered. Species **3** and **4** with attached methylmalonyl and propionyl residues were substantially more abundant than in the case of wild-type Module 1. These observations are consistent with the above-described experiments that indicated that H346 played a role in both chain translocation (Figure 5) and chain elongation (Table 1). The relatively low abundance of species **4** (as compared, for example, with the C211A mutant) and the virtual absence of species **5** also suggested that H346 stabilized the transition state associated with decarboxylation of the ACP-bound methylmalonyl extender unit. Analogously, the reduced level of species **5** along with the virtual absence of species **4** in the H384A mutant suggested a similar role for this His residue. In contrast, although the K379A mutant did not harbor significant quantities of either diketide product attached to its ACP domain (corresponding to Ppant species **5** or **6**), the observation of **4** implied that the mutant KS domain was capable of promoting decarboxylation of the ACP-bound methylmalonyl extender unit. Thus, K379 plays a role in formation of the C–C bond between the electrophilic and nucleophilic substrates. The most pronounced consequence of the S315A mutation was an increased abundance of **4**, consistent with a role for S315 in C–C bond formation. The

Q386E and N455L variants showed comparable distributions of ACP species relative to wild-type Module 1. The mechanistic implications of these findings are discussed below.

DISCUSSION

Ketosynthases, which catalyze the central chain-building decarboxylative Claisen condensation in both fatty acid and polyketide synthases, are the most evolutionarily conserved protein components in this superfamily of multifunctional enzymes. To our knowledge, this is the first report of enzymological investigation into the contributions of the most conserved residues in the active sites of the KS domains from an assembly line PKS. Below we summarize our key mechanistic findings.

Role of C211

As expected, the C211A mutant of Module 1 exhibited no turnover activity corresponding to diketide formation (Table 1), nor could the protein be labeled by [^{14}C]-propionate (Figure 5). Nonetheless, this mutant is still a competent methylmalonyl-ACP decarboxylase (Table 2). Robust decarboxylase activity has been previously observed for the C $\rightarrow$ A active site mutant of the *Streptococcus pneumoniae* FabF,⁵ but not in the analogous mutant of the vertebrate fatty acid synthase.¹⁶ Our data leads us to conclude that the main role of C211 is to provide an exceptionally nucleophilic active site for anchoring the growing polyketide chain in advance of the signature decarboxylative Claisen condensation.

H346 Enhances Both Intermodular Chain Translocation and Decarboxylation of Methylmalonyl-ACP

Steady state kinetic analysis demonstrated that H346 is essential for enzyme-catalyzed substrate turnover. Although intermodular chain translocation by the H346A mutant module is somewhat reduced (Figure 5), this mutation has a more pronounced effect on chain elongation (Table 1). We propose that H346 acts as a general acid to promote chain translocation by protonating the thiolate of the departing donor ACP of the LDD protein. It then also contributes to chain elongation by stabilizing the transition state associated with decarboxylation of methylmalonyl-ACP (Table 2), possibly by activation of the C-1 carbonyl of methylmalonyl-ACP. An analogous role has been proposed for the corresponding residue in the FabB ketosynthase of the *E. coli* fatty acid synthase⁴ as well as the KS domain of the vertebrate fatty acid synthase.³ Notably, however, whereas the equivalent H $\rightarrow$ A mutants of the vertebrate fatty acid synthase³ and FabF from *Streptococcus pneumoniae*⁵ retain measurable turnover activity, the H346A mutant of DEBS Module 1 is essentially inactive. This difference may reflect the enhanced turnover rates of fatty acid synthases as compared to assembly line PKSs and hence the greater dynamic range for detection of reduced activity.

H384 Contributes to Intermodular Chain Translocation and Decarboxylation of Methylmalonyl-ACP

The pronounced decrease in k_{cat}/K_{50} of H384 (Table 1) in addition to the moderate decrease in Module 1 acylation (Figure 5) indicates that this residue plays a role in chain translocation (Step 1, Figure 1), possibly by acting as a general base to increase nucleophilicity of the C211 thiol. Decarboxylation of methylmalonyl-ACP is also attenuated in this mutant (Table

2). This behavior is qualitatively consistent with that of the vertebrate fatty acid synthase,^{4,3,5} although the loss of activity due to the H → A mutation in the fatty acid synthase is considerably greater.³ An analogous mutation in the *E. coli* FabB renders the organism incapable of responding to a temperature change in vivo, which is a hallmark of the physiological role of FabB in *E. coli*.⁵ We propose that this invariant His residue in assembly line PKSs acts to initiate decarboxylation of the C-3 carboxylic acid of methylmalonyl-ACP through stereoelectronic effects in which ion pairing between the carboxylate and the H384 proton orients the scissile bond between the carboxylate and C-2 of the methylmalonyl substrate perpendicular to the plane of the adjacent acyl thioester, thereby allowing the requisite orbital overlap (Figure 6).

K379 Promotes C–C Bond Formation

The K379A mutant is, for the most part, inactive in turnover assays (Table 1), but is nonetheless capable of decarboxylating methylmalonyl-ACP (Table 2). Very low activities were also reported for the analogous mutants of the vertebrate fatty acid synthase³ and the *Streptococcus pneumoniae* FabF,⁵ although the precise basis for this loss of activity was not explored. Furthermore, K → A mutants in the *Streptococcus pneumoniae* FabF and the *Escherichia coli* FabB ketosynthases retained ACP → KS translocation efficiency, entirely consistent with our observation of chain translocation between the ACP domain of the LDD and the K379A mutant of DEBS Module 1 (Figure 5). We propose that K379 enhances the ability of the nucleophilic enol to attack the electrophilic acyl thioester, possibly by increasing the proton donating capabilities of H346 (Figure 6).

Other Active Site Mutants

The finding that the ACP domain of the S315A mutant of Module 1 acquires an atypically high level of propionate derived from decarboxylation of the methylmalonyl-derived substrate (Table 2) can be rationalized by the presence of this residue on a loop near the entry tunnel to the KS active site (Figure 1B). Mutagenesis-guided docking studies^{17–19} suggest that S315 interacts with the Ppant arms of the ACP domains involved in both chain translocation and chain elongation. By destabilizing both KS-ACP complexes, the S315A mutation could reduce the rate of either reaction. No effect on translocation is observed, however (Figure 5 and Table 1). We therefore suggest that the primary role of S315 is to stabilize methylmalonyl-ACP bound to the KS active site, thereby enabling tight coupling between decarboxylation and C–C bond formation. Aberrant coupling would be expected to lead to accumulation of higher levels of ACP-bound propionate due to protonation of the transiently generated derived enolate.

The marked reduction in k_{cat}/K_{50} of the Q386E mutant of Module 1 (Table 1) is not as easily explained, since this residue is not expected to interact directly with the ACP. Interestingly, the Gln residue is highly conserved across iterative PKSs, whereas the corresponding residue is not conserved in assembly line PKSs (Figure 2) and is in close proximity to H384. We propose that Q386 enhances the basicity of H384, similar to the catalytic triads of serine and cysteine proteases. The observed increase in k_{cat}/K_{50} for T209A can be rationalized based on its proximity to C211. It is possible that a smaller residue at this position enhances the accessibility of the Ppant arm of LDD-ACP.

The observation that the N455L mutation results in >50% maximal occupancy of Module 1 is intriguing, given our previous finding that simultaneous occupancy by growing polyketide chains of the KS and ACP domains of a wild-type module is precluded.² N455 is located in a β -sheet adjacent to F449, a highly conserved residue whose amide hydrogen is thought to stabilize the tetrahedral intermediate during chain translocation.⁵ Indeed, F449 was deemed a “gatekeeper”, controlling the reaction order and directing the incoming acyl chain into the substrate binding pocket of the KS.⁵ Perturbations to the β -sheet may affect the timing of chain translocation, resulting in a higher occupancy level. Future studies directed toward understanding the strict control that a KS active site has over its acylation state should therefore focus on F449.

Proposed Catalytic Mechanism of a KS Domain of an Assembly Line PKS

Taken together, the above experiments and analyses lead us to propose the mechanism outlined in Figure 6 for the translocation and elongation half-reactions catalyzed by the KS domains of assembly line PKSs. Notably, this mechanism implies that H346 plays a critical role in C–C bond formation, a departure from previous characterizations of this residue in fatty acid synthases.^{3–5} We have also identified hitherto overlooked residues proximal to the active site Cys that play important roles in translocation (Q386), C–C bond formation (S315), or KS acylation (N455). More generally, this model provides a well-defined starting point for further in-depth analysis of a signature PKS domain. Of particular interest is the coupling between chain elongation and vectorial chain translocation, as a growing polyketide chain alternates between successive KS and ACP domains in assembly line biosynthesis.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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ABBREVIATIONS

PKS	Polyketide Synthase
KS	a β -ketoacyl ACP synthase
DEBS	6-Deoxyerythronolide B Synthase
M1	DEBS Module 1
ACP	Acyl Carrier Protein

LDD	Loading DiDomain
Ppant	Phosphopantetheine
AT	Acyltransferase
KR	ketoreductase
TE	Thioesterase

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Figure 1.

(A) Catalytic cycle of DEBS Module 1. The cycle begins in step (I) with the translocation of a propionyl primer from the Loading DiDomain (LDD) onto a Cys residue in the ketosynthase domain (KS) of Module 1. Transacylation (II) by the acyltransferase (AT) loads a methylmalonyl extender unit onto the ACP. This is followed by a decarboxylative Claisen condensation, resulting in an unreduced diketide chain whose stereochemistry is then set by the ketoreductase (KR), (Elongation and Reduction, III). The cycle returns to its starting position after downstream translocation (IV) of the polyketide chain onto the active site Cys of Module 2. (B) Model of the active site of the KS domain from Module 1. The model was generated by homology with KS3⁸ using iTasser. Residues of interest in this study are highlighted (active site Cys is orange, His-His-Lys triad is in green, other residues are in cyan).

Figure 2.

Sequence alignment of the noniterative DEBS KS1 with the iterative Lovastatin B (LovB) KS. Those residues in red color for DEBS KS1 are 90% conserved across 226 noniterative PKS-KSs and those in red for the LovB KS are 90% conserved across 24 iterative PKS-KSs. Black boxes indicated residues that fall within 10 Å of the active site Cys as judged by the crystal structure of the KS-AT fragment of DEBS Module 3.⁸ Residues highlighted in blue were chosen for mutagenesis in this study. Sequences were downloaded from Clustermine360 and consensus sequence and alignments were generated using MView and MUSCLE from the European Bioinformatics Institute.

Figure 3.

Steady state kinetic analysis of wild-type and mutant Module 1 proteins. (Left) Assay setup utilizes the previously described bimodular system, which generates compound **1** in the presence of NADPH, propionyl-CoA, and methylmalonyl-CoA. (Right) $V_o/[E_o]$ versus [LDD] data with the identity of each mutant protein shown in a different color. (Right). Error bars for data shown are $\pm$ s.d. for $n = 3$. For experimental details, see Materials and Methods.

Figure 4.

Verification of product identity from PKS assays in Figure 3: Extracted ion chromatograms (EICs) of **1** ($M + H - H_2O = 155.1063$) for all 11 proteins used in this study in the presence of equimolar concentrations of LDD and Module 2+TE, and excess NADPH, propionyl-CoA, and methylmalonyl-CoA. For all species identified the expected $M+H-H_2O$, $M+H$, and $M+Na$ fragmentation pattern was found, with the exception of H346A*. The EIC for K379A** was obtained with the mutant in 10× excess compared to LDD and Module 2+TE.

Figure 5.

Occupancy of wild-type and mutant Module 1 proteins under single turnover conditions: Each Module 1 protein was incubated with equimolar LDD and excess ^{14}C -propionyl-CoA, methylmalonyl-CoA, and NADPH. Radioactive decay counts were converted into % occupancy using the standard curve, as described previously.²

Figure 6.
Proposed catalytic mechanism of a KS Domain of an assembly line PKS: Residues in red are highlighted as important in the corresponding catalytic step.

Scheme 1. Sources of Species Bound to the Ppant Arm of the ACP Domain of Wild-Type and Mutant Module 1 Proteins^a

^aLDD and Module 1 were incubated with a mixture of butyryl-CoA, methylmalonyl-CoA, and NADPH. After 15 min, the reaction was proteolyzed and Ppant ejection analysis was performed on a QTrap mass spectrometer. Five species, ACP-N-ethylmaleimide (**2**), methylmalonyl-ACP (**3**), propionyl-ACP (**4**), propionyl-derived diketidyl-ACP (**5**), and butyryl-derived diketidyl-ACP (**6**), were identified, and their relative abundance was quantified. For experimental details, see Materials and Methods.

Table 1Steady State Kinetic Parameters for Wild-Type and Mutant Module 1 Proteins^a

mutant	k_{cat} 1 (min ⁻¹)	K_{50} (μM)	k_{cat}/K_{50} ($\mu\text{M}^{-1} \text{min}^{-1}$)
WT	2.5 $\pm$ 0.1	0.09 $\pm$ 0.02	28 $\pm$ 6.3
H346A	N/A	N/A	N/A
H384A	0.4 $\pm$ 0.1	0.09 $\pm$ 0.02	4.4 $\pm$ 1.5
K379A	N/A	N/A	N/A
C211A	N/A	N/A	N/A
N455L	2.3 $\pm$ 0.1	0.08 $\pm$ 0.01	29 $\pm$ 3.8
N381A	2.3 $\pm$ 0.1	0.06 $\pm$ 0.01	38 $\pm$ 6.6
Q386E	1.7 $\pm$ 0.1	0.24 $\pm$ 0.07	7.1 $\pm$ 2.1
H457A	1.9 $\pm$ 0.1	0.08 $\pm$ 0.02	24 $\pm$ 6.1
T209A	2.9 $\pm$ 0.1	0.06 $\pm$ 0.01	48 $\pm$ 8.2
S315A	2.4 $\pm$ 0.1	0.11 $\pm$ 0.01	22 $\pm$ 2.2

^aUsing the assay depicted in Figure 3, k_{cat} for the formation of **1** and K_{50} between the LDD and M1 was determined. Values reported were rounded to the nearest tenth and error shown is $\pm$ s.e. for $n = 3$.

Table 2

Relative Occupancy of the ACP Domains of Wild-Type and Selected Mutants of DEBS Module 1^a

compound	2	3	4	5	6
WT	23	3	3	28	43
H346A	55	9	15	6	15
H384A	37	7	5	13	39
K379A	46	26	20	4	4
C211A	13	2	84	0	0
N455L	13	3	2	25	57
Q386E	22	11	2	17	48
S315A	16	2	19	24	40

^aValues represent the percent of the total ACP species as assessed by ion counts, with data rounded to the nearest unit percentage. Data reported are the mean of three separate runs, with the s.e. not exceeding 10% for any of the conditions reported. For experimental details, see Materials and Methods.