Reconstituting Modular Activity from Separated Domains of 6-Deoxyerythronolide B Synthase †

ARTICLE *in* BIOCHEMISTRY · DECEMBER 2004

Impact Factor: 3.02 · DOI: 10.1021/bi048418n · Source: PubMed

6 AUTHORS, INCLUDING:

Chu-Young Kim
National University of Singapore
19 PUBLICATIONS **874** CITATIONS
SEE PROFILE

Yinyan Tang
University of California, San Francisco
12 PUBLICATIONS **568** CITATIONS
SEE PROFILE

Reconstituting Modular Activity from Separated Domains of 6-Deoxyerythronolide B Synthase†

Chu-Young Kim,[‡,§] Viktor Y. Alekseyev,[§] Alice Y. Chen,[‡] Yinyan Tang,[§] David E. Cane,[||] and Chaitan Khosla*[,‡,§,⊥]

Departments of Chemical Engineering, Chemistry, and Biochemistry, Stanford University, Stanford, California 94305-5025, and Department of Chemistry, Brown University, Box H, Providence, Rhode Island 02912-9108

Received July 25, 2004; Revised Manuscript Received September 21, 2004

ABSTRACT: The hallmark of a type I polyketide synthase (PKS), such as the 6-deoxyerythronolide B synthase (DEBS), is the presence of catalytic modules comprised of covalently fused domains acting together to catalyze one round of chain elongation. In addition to an obligate ketosynthase (KS), acyl transferase (AT), and acyl carrier protein (ACP), a module may also include a ketoreductase (KR), dehydratase (DH), and/or enoyl reductase (ER) domain. The size, flexibility, and fixed domain−domain stoichiometry of these PKS modules present challenges for structural, mechanistic, and protein-engineering studies. Here, we have harnessed the power of limited proteolysis and heterologous protein expression to isolate and characterize individual domains of module 3 of DEBS, a 150-kD protein consisting of a KS, an AT, an ACP, and an inactive KR domain. Two interdomain boundaries were identified via limited proteolysis, which led to the production of a 90-kD KS-AT, a 142-kD KS-AT-KR^0, and a 10-kD ACP as structurally stable stand-alone proteins. Each protein was shown to possess the requisite catalytic properties. In the presence of the ACP, both the KS-AT and the KS-AT-KR^0 proteins were able to catalyze chain elongation as well as the intact parent module. Separation of the KS from the ACP enabled direct interrogation of the KS specificity for both the nucleophilic substrate and the partner ACP. Malonyl and methylmalonyl extender units were found to be equivalent substrates for chain elongation. Whereas ACP2 and ACP4 of DEBS could be exchanged for ACP3, ACP6 was a substantially poorer partner for the KS. Remarkably, the newly identified proteolytic sites were conserved in many PKS modules, raising the prospect of developing improved methods for the construction of hybrid PKS modules by engineering domain fusions at these interdomain junctions.

Structural diversity and broad biological activity are hallmarks of the polyketide natural product family (*1*). These complex structures are synthesized in nature from a limited repertoire of simple carboxylic acids by multifunctional proteins called polyketide synthases (PKSs).[1] Building blocks are incorporated sequentially by a PKS to synthesize the desired natural product. A PKS minimally requires three functional proteins to perform one round of chain elongation: a ketosynthase (KS), an acyl transferase (AT), and an acyl carrier protein (ACP). The growing polyketide chain is anchored on the KS via a thioester linkage. Once the AT has transferred an acyl-CoA extender unit to the phosphopantetheine arm of the ACP, the KS catalyzes decarboxylative condensation between the electrophile (growing chain) and the nucleophile (extender unit) to form a β-ketoacyl-ACP intermediate. In modular PKSs, functional units such as the KS, AT, and ACP are referred to as domains, whereas modules refer to collections of catalytic domains that act together to complete one chain elongation cycle. A PKS module may contain, in addition to the three core domains described above, modifying domains such as a β-ketoreductase (KR), a dehydratase (DH), and/or an enoyl reductase (ER). The KR reduces the β-ketothioester intermediate generated by the KS to a β-hydroxythioester. The DH dehydrates the resulting intermediate into an α,β-unsaturated ester, whereas ER hydrogenates the olefin to yield a fully reduced β-carbon atom. Thus, the KR, DH, and ER domains expand the functional group diversity of the final polyketide natural product. Two main categories of PKSs are found in microorganisms. Type I PKSs have covalently linked domains, such that one or more modules are contained on a single, large polypeptide chain. In contrast, type II PKSs have individually expressed domains.

The 6-deoxyerythronolide B synthase (DEBS) from *Saccharopolyspora erythraea* is a multimodular type I PKS that catalyzes the biosynthesis of the macrocyclic core of the antibiotic erythromycin (Figure 1A) (*2*, *3*). Many important biochemical and mechanistic features of DEBS catalysis have been elucidated over the past decade (*4*). Genetic manipulation of DEBS has also yielded numerous 6-deoxyerythronolide B analogues by domain inactivation, insertion, or

† This research was supported by Grants from the National Institutes of Health (CA 66736 to C.K. and GM 22172 to D.E.C.).

* To whom correspondence should be addressed. Phone/Fax: (650) 723-6538. E-mail: ck@chemeng.stanford.edu.

‡ Department of Chemical Engineering, Stanford University.

§ Department of Chemistry, Stanford University.

|| Department of Chemistry, Brown University.

⊥ Department of Biochemistry, Stanford University.

[1] Abbreviations: PKS, polyketide synthase; DEBS, 6-deoxyerythronolide B synthase; M3TE, module 3+thioesterase; KS, ketosynthase; AT, acyl transferase; KR, β-ketoreductase; DH, dehydratase; ER, enoyl reductase; ACP, acyl carrier protein; TE, thioesterase; NDK, natural diketide; HSNAc, *N*-acetylcysteamine.

10.1021/bi048418n CCC: $27.50
Published on Web 10/09/2004

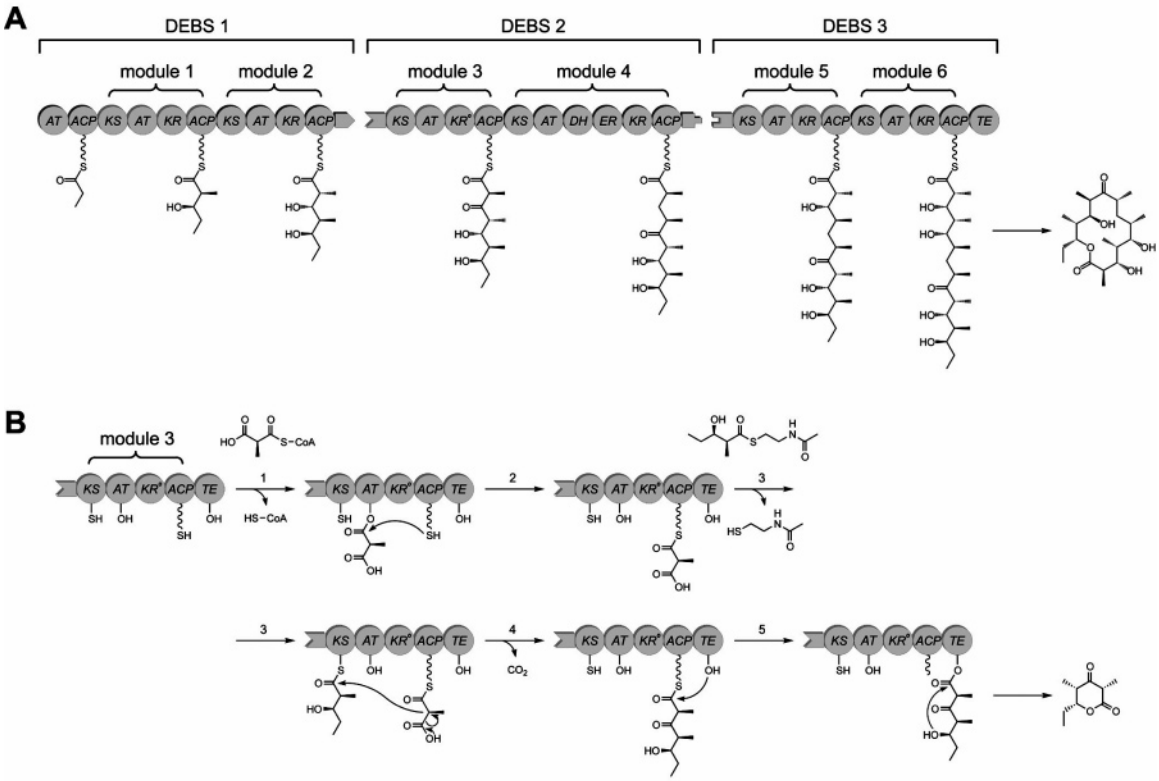


FIGURE 1: (A) Modular organization of DEBS. Each catalytic domain is represented by a circle; KS, AT, ACP, DH, ER, KR, and TE. KR^0 denotes an inactive ketoreductase domain. The phosphopantetheine prosthetic group of ACP is drawn as a curly line. Interprotein linkers are shown as matching tabs. (B) Chain elongation cycle catalyzed by DEBS M3TE. AT is acylated with a methylmalonyl extender unit from its CoA derivative, which gets transferred to the downstream ACP. KS is primed with a 2-methyl-3-hydroxy pentanoyl unit from NDK-SNAc. Condensation takes place in the active site of KS with the release of carbon dioxide. TE catalyzes lactone formation with the release of the final product.

substitution (*5*). In contrast, surprisingly little is known about the structure of DEBS or the structure of type I PKSs in general. The X-ray structures of the isolated TE domain from DEBS and the pikromycin PKS have recently been reported (*6*−*7*), and the solution NMR structures of terminal-docking domains of DEBS2 and DEBS3 have also been determined (*8*). However, the absence of high-resolution structural insights into any other DEBS domains or modules (or for that matter, those of any other type I PKS proteins) presents a major stumbling block for the enzymology and protein engineering of these remarkable modular megasynthases. As a step in this direction, we investigate the effect of splitting a module into its constituent domains. Our results have encouraging implications for the structural biology of PKSs as well as for combinatorial biosynthesis of new polyketide natural products.

MATERIALS AND METHODS

Reagents and Chemicals. DL-[2-Methyl-^{14}C]-methylmalonyl-CoA and [2-^{14}C]-malonyl-CoA were from American Radiolabeled Chemicals. [^{14}C]-NDK-SNAc was from Amersham Pharmacia. All other chemicals, including nonlabeled CoA derivatives, were from Sigma. Thin-layer chromatography (TLC) plates (IB2) were from J. T. Baker. SDS−PAGE-gradient gels (4−15% acrylamide) were from BioRad. Ni-NTA affinity resin was from Qiagen. HiTrap-Q anion exchange and Superdex-200 size-exclusion columns were from Amersham Pharmacia.

Expression and Purification of Module 3+Thioesterase (M3TE). M3TE was purified according to published procedures (*9*). Plasmid pRSG34 (*10*) was coexpressed in *Escherichia coli* with a plasmid encoding the Sfp phosphopantetheinyl transferase to ensure complete pantetheinylation of the ACP domain of M3TE (*11*). Transformed *E. coli* cells were grown in LB medium at 37 °C to an $OD_{600} = 0.6$ before the cultures were cooled to 30 °C and induced with 0.2 mM isopropyl-β-D-galactopyranoside (IPTG) for 12 h. Cells were harvested by centrifugation (4500*g*, 15 min), resuspended in lysis/wash buffer (50 mM phosphate at pH 7.6, 300 mM NaCl, and 10 mM imidazole), lysed with sonication (5 × 1 min), and cellular debris was removed by centrifugation. Nickel-NTA agarose resin was added directly to the supernatant (8 mL of resin/L of culture) and mixed for 20 min at 4 °C. This resin was poured into a fritted column, washed with 10 column volumes of lysis/wash buffer, and eluted with 3 column volumes of elution buffer (50 mM phosphate at pH 7.6, 100 mM NaCl, and 150 mM imidazole). The eluted protein was applied to a HiTrap-Q anion-exchange column and eluted with an increasing linear NaCl gradient. M3TE eluted at approximately 370 mM NaCl (Figure 2A).

Preparation of Proteolytic Fragments. Purified M3TE (1 mg/mL in 20 mM HEPES at pH 7.6) was digested with 1 μg/mL trypsin at 4 °C and quenched after 10 min by addition of 6 mM benzamidine. Ni-NTA agarose was added to the mixture and stirred for 30 min at 4 °C. The resin was subsequently removed using a gravity column and discarded. The eluate was applied to a HiTrap-Q anion-exchange column and eluted with 350 mM NaCl. The large fragment was further purified via gel-filtration chromatography using

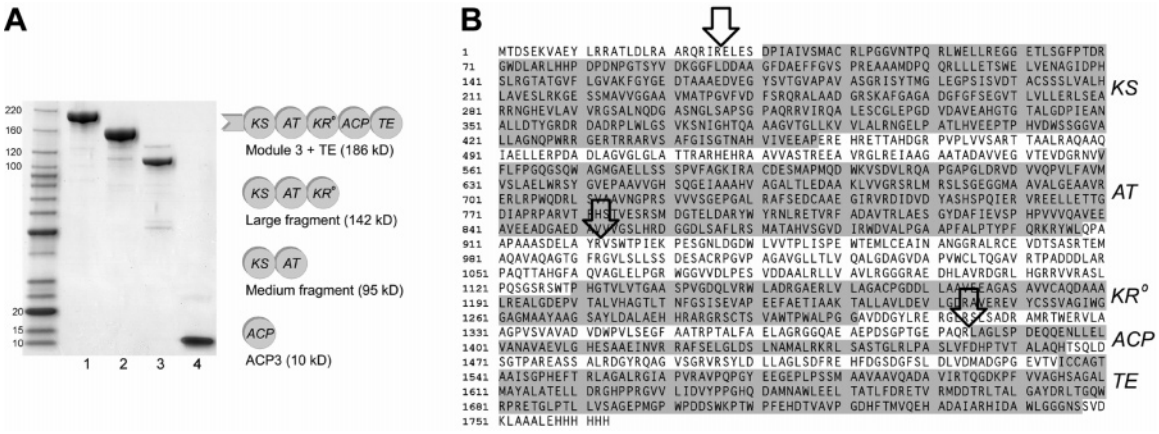


FIGURE 2: (A) SDS−PAGE of purified DEBS module 3 proteins. Lane 1, recombinant M3TE; lane 2, 142-kD proteolytic fragment of M3TE; lane 3, 95-kD proteolytic fragment of M3TE; and lane 4, recombinant ACP3. Molecular weight of the proteolytic fragments was determined by ESI−MS. (B) Amino acid sequence of M3TE. Individual domains, as identified by sequence homology with fatty acid synthase, are shaded in gray and are labeled on the right. Arrows indicate experimentally observed trypsin partial proteolysis sites. The N terminus of each of the two proteolytic fragments was determined by N-terminal sequencing. The C terminus of each protein was deduced from the ESI−MS-determined molecular weight.

a Superdex-200 column. To generate the medium fragment, purified M3TE was digested with trypsin for 1 h under identical conditions. The purification procedure for the medium fragment was identical to that for the large fragment. Both the large and medium fragments were characterized by mass spectrometry (ESI−MS) and N-terminal sequencing (automated Edman degradation). The observed molecular masses of the large and medium fragments were 142.58 and 94.73 kD, respectively (calcd masses were 142.24 and 94.56 kD, respectively).

Expression and Purification of ACPs. DNA manipulations were performed using standard protocols (*12*). The DNA sequence encoding DEBS ACP3 (nucleotides 4168−4440; GenBank accession no. M63677) was amplified by PCR as an *Nde*I−*Eco*RI fragment using primers 5′-GAGCCG-CATATG**CGGCTCGCGGGGCTTTCC**-3′ and 5′-CGCG-GCGAATTCTTA**GGCGTCACCGACGAGCCGGGC**-3′ (sequences complementary to DEBS are shown in bold), with a stop codon inserted at the end of the region of homology (delineated by alignment to other ACP domains of DEBS). This *Nde*I−*Eco*RI fragment was cloned into a pET28a expression vector. The resulting plasmid pVYA05, encoding an N-terminal His_6-tagged ACP, was used to produce ACP3 in *E. coli* BL21(DE3). The DNA sequence encoding DEBS ACP6 was similarly amplified using primers 5′-TTCATAT**GGCGGCCCCGGCGCGGG**-3′ and 5′-T-TGAATTCA**GAGCTGCTGTCCTATGTGGTCG**-3′. The resulting *Nde*I−*Eco*RI fragment was cloned into pET28a to yield plasmid pPK223, encoding DEBS ACP6 with an N-terminal His_6 tag, The construction of genes encoding ACP2 (pNW7) and ACP4 (pNW8) has been previously described (*13*, *14*). All ACPs were expressed and purified using the following procedure. Cells were grown at 37 °C in LB medium with 100 μg/mL kanamycin to an OD_{600} = 0.6, then cooled to 25 °C, and induced with 0.2 mM IPTG for 12 h. The cells were harvested by centrifugation and disrupted via sonication. ACPs were purified using Ni-affinity chromatography. The resulting protein was applied to a HiTrap-Q anion-exchange column and recovered in the flow-through. A typical protein yield was 10 mg/L culture volume.

[^{14}C]Methylmalonyl- and [^{14}C]Malonyl-CoA Labeling. KS-AT-KR^0 (20 μM, in 100 mM phosphate at pH 7.2) alone or KS-AT-KR^0 together with holo-ACP3 (20 and 100 μM, respectively, in 100 mM phosphate at pH 7.2) were incubated on ice with 100 μM [^{14}C]methylmalonyl-CoA or [^{14}C]-malonyl-CoA for 5 min. Similarly, KS-AT (20 μM, in 100 mM phosphate at pH 7.2) alone or KS-AT together with holo-ACP3 (20 and 100 μM, respectively, in 100 mM phosphate at pH 7.2) were incubated on ice with 100 μM [^{14}C]methylmalonyl-CoA or [^{14}C]malonyl-CoA for 5 min. Samples were quenched with SDS−PAGE loading buffer lacking any reducing agents such as dithiothreitol or β-mercaptoethanol and loaded directly onto a SDS−PAGE gel. The gel was dried using a BioRad gel-drying system and analyzed using a Packard phosphorimager.

[^{14}C]NDK-SNAc Labeling. KS-AT-KR^0 (20 μM, in 100 mM phosphate at pH 7.2) and KS-AT (20 μM, in 100 mM phosphate at pH 7.2) were each incubated with 1 mM [^{14}C]-NDK-SNAc alone or together with 1 mM cerulenin for 10 min at room temperature. Samples were quenched with SDS−PAGE loading buffer lacking any reducing agents and loaded directly onto a SDS−PAGE gel. The gel was dried using a BioRad gel-drying system and analyzed using a Packard phosphorimager.

Triketide Lactone Formation. The reaction volume was 10 μL for all single time point assays. As a positive control, M3TE (20 μM, in 100 mM phosphate at pH 7.6) was incubated with 1 mM [1-^{14}C](2*S*,3*R*)-2-methyl-3-hydroxypentanoyl-SNAc (NDK-SNAc) for 15 min at room temperature, followed by addition of methylmalonyl-CoA (1 mM final concentration). The reaction was quenched after 120 min by adding 20 μL of 0.5 M potassium hydroxide and heating the mixture for 20 min at 65 °C. A total of 10 μL of 1.5 M hydrochloric acid was added, and the mixture was dried in a Speed-vac for 1 h. The pellet was resuspended in 10 μL of ethyl acetate and spotted onto a TLC plate. A 60:40 mixture of ethyl acetate/hexane was used for radioactive TLC and visualized using a Packard phosphorimager. As a control, KS-AT-KR^0 or KS-AT (20 μM, in 100 mM phosphate at pH 7.6) was preincubated with 1 mM [^{14}C]-NDK-SNAc for 15 min at room temperature, followed by

addition of holo-ACP3 (100 μM, in 100 mM phosphate at pH 7.6) and subjected to the same procedures as above. To assay triketide lactone formation by M3TE, tryptic fragments and recombinant module 3 KS-AT didomain, methylmalonyl- or malonyl-preloaded ACP2, ACP3, ACP4, and ACP6 (100 μM, in 100 mM phosphate at pH 7.6) were used. It should be noted that M3TE catalyzes multiple turnovers, whereas KS-AT together with ACP catalyzes a single turnover.

Expression and Purification of KS-AT Didomain from DEBS Module 3. The DNA sequence encoding the KS-AT didomain from pRSG34 was modified by eliminating the sequences encoding the domains downstream of a conserved YRVXW sequence (Figure 2B). The resulting plasmid, pAYC2, was transformed into BL21(DE3) *E. coli* cells. Cells were grown at 37 °C in LB medium with 100 μg/mL carbenicillin to an $OD_{600} = 0.6$, at which point they were cooled to 18 °C and induced with 0.5 mM IPTG for 24 h. The cells were harvested by centrifugation and disrupted by sonication. The KS-AT didomain was purified using Ni-affinity and anion-exchange chromatography as described above for the purification of proteolytic fragments. The final yield of the protein was 20 mg/L.

RESULTS

Module 3 of DEBS was chosen as a target for this study because its molecular recognition features have been extensively investigated (*9*, *10*) and because it represents the simplest kind of PKS module comprising only of a functional KS, AT, and ACP domain. Although this module possesses a domain homologous to catalytically active KR domains (*3*), on the basis of the known structure of 6-deoxyerythronolide B as well as detailed sequence analysis, it was expected that the domain, designated KR^0 hereafter, is inactive.

Post-translational Modification of ACP2, ACP3, ACP4, and ACP6. Acyl carrier proteins of polyketide synthases require covalent attachment of a 4′-phophopantetheine moiety from coenzyme A onto a conserved serine residue to become catalytically active (*11*). The holo forms of ACPs were prepared by coexpression of the Sfp phosphopantetheinyl transferase gene from *Bacillus subtilis* in *E. coli*. Alternatively, *in vitro* treatment of apo-ACPs with either methylmalonyl-CoA or malonyl-CoA substrates in the presence of recombinant Sfp yielded methylmalonyl- or malonyl-ACP, as verified by ESI−MS.

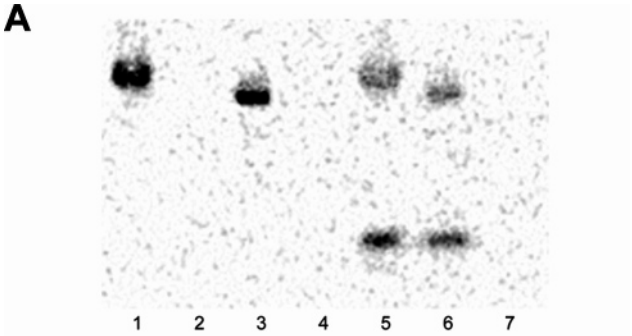
Limited Proteolysis of M3TE. M3TE was digested with trypsin at 4 °C as described in the Materials and Methods. SDS−PAGE analysis of the digestion mixture revealed a large size fragment (142 kD) and a medium size fragment (95 kD). Neither fragment bound to nickel-affinity resin, suggesting that the C-terminal His_6 tag of the parent M3TE molecule had been removed. The two fragments were separated using anion-exchange chromatography and further purified using gel-filtration chromatography to yield highly pure proteins (lanes 2 and 3 of Figure 2A). Both proteins appear to be monomeric in solution as determined by native PAGE analysis. Purified fragments were stable and showed no indication of degradation after 1 week when stored at 4 °C. The high purity of these proteins allowed successful mass spectrometric and N-terminal sequencing analysis. The N-terminal sequences were used to identify the start of each fragment, and the molecular weights were used to deduce the size and infer the presumed C-terminal cleavage site (Figure 2B). The 142-kD fragment is composed of the KS-AT-KR^0 domains, while the 95-kD fragment is composed of the KS-AT region of module 3. It was encouraging to note that these trypsin recognition sites were located exclusively in the interdomain linker regions, and the resulting fragments contained full-length domains.

Expression and Purification of Recombinant KS-AT Didomain. On the basis of the M3TE proteolysis results, an expression plasmid, pAYC2, encoding the DEBS module 3 KS-AT didomain was constructed. The resulting plasmid expressed a soluble protein of 98 kD at a yield of 20 mg/L culture (Figure 5A). In all of the experiments described below, this recombinant protein was indistinguishable from the KS-AT didomain protein isolated via limited proteolysis of M3TE.

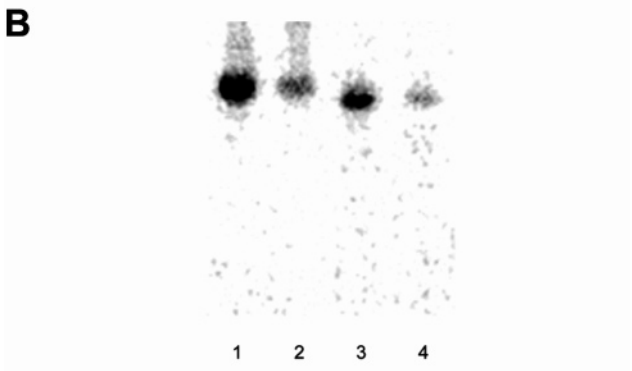
AT Activity. The AT domain transfers polyketide chain extension units from methylmalonyl-CoA to the ACP (Figure 1B). In the DEBS system, such extender AT domains are highly specific for the methylmalonyl-CoA substrate (*15*). The KS-AT-KR^0 and KS-AT proteins prepared by limited proteolysis of M3TE were incubated with [^{14}C]methylmalonyl-CoA or [^{14}C]malonyl-CoA, and protein labeling was visualized by SDS−PAGE followed by phosphorimaging. Both KS-AT-KR^0 and KS-AT were labeled by [^{14}C]methylmalonyl-CoA (lanes 1 and 3 of Figure 3A, respectively) but not with [^{14}C]malonyl-CoA (lanes 2 and 4 of Figure 3A, respectively), indicating that the AT domain maintained acylation activity and substrate specificity outside the context of an intact module. Furthermore, this AT activity did not appear to be affected by the presence or absence of an adjacent KR^0 domain. When holo-ACP3 was present in the reaction mixture, the [^{14}C]methylmalonyl label was transferred from KS-AT-KR^0 and KS-AT to the ACP (lanes 5 and 6 of Figure 3A, respectively), clearly demonstrating that the AT domains within the fragments were fully functional.

KS Acylation Activity. The KS domain receives the growing polyketide chain from the ACP of the immediate upstream module onto a conserved KS cysteine residue via a thioester linkage. To test whether this acylation activity is maintained, the KS-AT-KR^0 and KS-AT fragments were incubated with NDK-SNAc, a known high-affinity substrate for DEBS module 3 (*9*, *10*). SDS−PAGE and phosphorimaging showed that both proteins were labeled (lanes 1 and 3 of Figure 3B), indicating efficient loading of the electrophilic substrate onto the KS domain. Labeling was substantially weaker when incubations were carried out in the presence of cerulenin (a KS-specific inhibitor) (lanes 2 and 4 of Figure 3B). As with acyl transferase activity, this activity also appeared unaffected by the presence and absence of the adjacent KR^0 domain.

Condensation Activity. In the final step of chain elongation, the acyl-KS (anchoring the growing chain) and acyl-ACP (anchoring the extender unit) interact to form the C−C bond by decarboxylative condensation, thereby extending the polyketide backbone by two carbon atoms. A radio-TLC assay was used to detect formation of the condensation product. In this assay, the KS-AT-KR^0 was preincubated with NDK-SNAc to ensure sufficient charging with the electrophilic substrate and then mixed with ACP preloaded with either a methylmalonyl or malonyl group. An aliquot of the



Lane	Protein	[^{14}C] substrate
1	KS-AT-KR^0	methylmalonyl-CoA
2	KS-AT-KR^0	malonyl-CoA
3	KS-AT	methylmalonyl-CoA
4	KS-AT	malonyl-CoA
5	KS-AT-KR^0 + ACP3	methylmalonyl-CoA
6	KS-AT + ACP3	methylmalonyl-CoA
7	ACP3	methylmalonyl-CoA



Lane	Protein	[^{14}C] substrate	Inhibitor
1	KS-AT-KR^0	NDK-SNAc	-
2	KS-AT-KR^0	NDK-SNAc	1 mM cerulenin
3	KS-AT	NDK-SNAc	-
4	KS-AT	NDK-SNAc	1 mM cerulenin

FIGURE 3: (A) [^{14}C]Methylmalonyl-CoA and [^{14}C]malonyl-CoA labeling of KS-AT-KR^0, KS-AT, and ACP3. (B) [^{14}C]NDK-SNAc labeling of KS-AT-KR^0 and KS-AT.

reaction mixture was withdrawn at various time points and quenched with base, resulting in the release of the expected ketolactones, which can be detected by TLC followed by phosphorimaging (parts A and B of Figure 4).

Specificity of the KS for Alternative ACP Partners. In type I PKSs, the KS and ACP domains that collaborate during chain elongation are invariably covalently attached. Consequently, it has not been possible to determine whether substitution of a given ACP domain with a heterologous counterpart carries a kinetic penalty. To test this hypothesis, condensation assays were performed using the KS-AT didomain protein and methylmalonyl-ACP2, -ACP3, -ACP4, and -ACP6. ACP2, ACP4, and ACP6 were expressed as apo proteins and modified using the Sfp protein following the same procedures described for ACP3 in the Results. The reaction product formation was visualized using TLC and

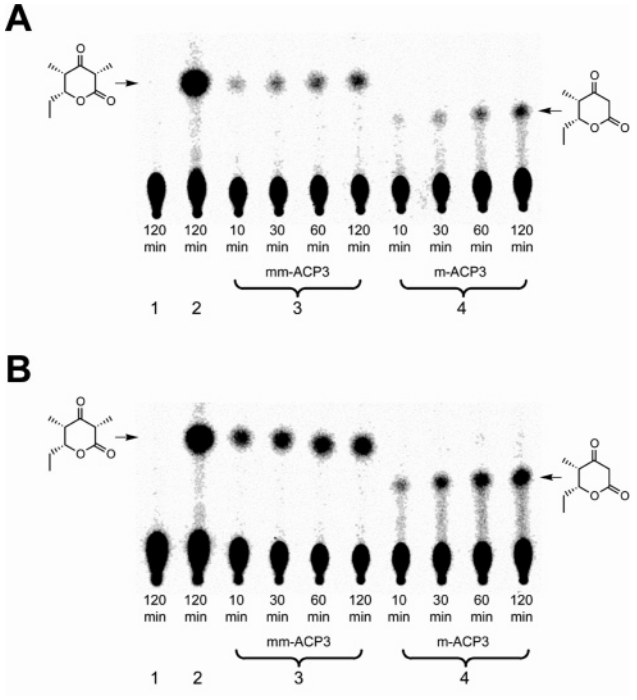


FIGURE 4: TLC phosphorimaging of triketide ketolactone formation. (A) Triketide ketolactone formation by KS-AT-KR^0. Lane 1, negative control reaction (KS-AT-KR^0, holo-ACP3, and [^{14}C]NDK-SNAc); lane 2, positive control reaction (M3TE, methylmalonyl-CoA, and [^{14}C]NDK-SNAc); lane 3, KS-AT-KR^0, methylmalonyl-ACP3, and [^{14}C]NDK-SNAc; lane 4, KS-AT-KR^0, malonyl-ACP3, and [^{14}C]NDK-SNAc. (B) Triketide ketolactone formation by KS-AT. Lane 1, negative control reaction (KS-AT, holo-ACP3, and [^{14}C]NDK-SNAc); lane 2, positive control reaction (M3TE, methylmalonyl-CoA, and [^{14}C]NDK-SNAc); lane 3, KS-AT, methylmalonyl-ACP3, and [^{14}C]NDK-SNAc; lane 4, KS-AT, malonyl-ACP3, and [^{14}C]NDK-SNAc. The TLC solvent system was 60:40 ethyl acetate/hexane.

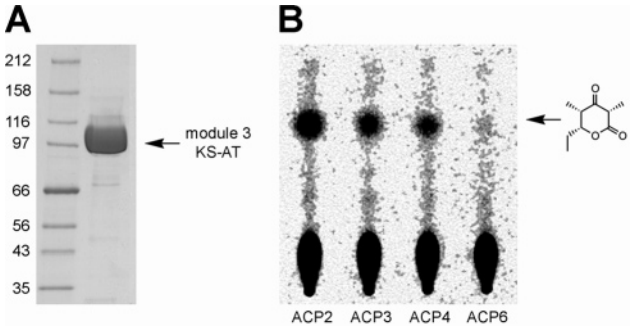


FIGURE 5: (A) SDS−PAGE of purified recombinant KS-AT didomain from DEBS module 3. (B) Triketide lactone formation by module 3 KS-AT didomain with ACP2, ACP3, ACP4, and ACP6.

phosphorimaging. As seen in Figure 5, whereas ACP2 and ACP4 were comparable substrates to ACP3, ACP6 was a significantly poorer substrate. Similar results were obtained with the KS-AT-KR° protein or if malonyl-ACPs were used instead of methylmalonyl-ACPs (data not shown). These results suggest that some ACP domains are more interchangeable than others.

DISCUSSION

Structure-based protein engineering of multifunctional PKSs represents an exciting scientific and technological

opportunity. At the heart of every PKS lies a combination of one (or more) KS, AT, and ACP component. These PKS components occur as stand-alone proteins in type II PKSs. High-resolution structures of individual KS, AT, and ACP protein subunits from type II PKSs have recently been obtained (*16*−*21*). In contrast, the structural biology of multidomain type I PKSs has lagged behind, presumably because of the large size and high flexibility of the corresponding modular proteins. Earlier attempts to express individual functional domains of PKS modules as stand-alone proteins have been unsuccessful, resulting in insoluble inclusion bodies (unpublished results). Here, we have taken a different approach, using limited proteolysis to identify structurally and functionally intact protein subunits within a single PKS module via limited proteolysis. There is considerable precedent for such an approach in the biochemistry of other multifunctional enzymes (*22*). Indeed, limited proteolysis has already led to valuable insights into the properties of multimodular PKS proteins such as DEBS1 (*23*) and the related vertebrate fatty acid synthase (*24*).

In the current study, limited trypsin-catalyzed proteolysis of DEBS module 3 has yielded two interesting multidomain fragments, a 142-kD KS-AT-KR^0 and a 95-kD KS-AT fragment. Although both proteins possess the same catalytically active domains, they have notably different structural properties. The former protein includes the large intervening linker region found in most PKS modules between N-terminal domains (KS, AT, and DH, when present) and C-terminal domains (ACP and, when present, ER and KR). It also includes the inactive KR^0 domain of module 3. Both these regions are missing from the smaller 95-kD fragment. It should be noted, however, that the C terminus of the 95-kD fragment maps to a short conserved sequence, YRVXW (Figure 2B), that lies downstream of not just the AT domain but also the DH domain whenever present. Together, these results suggest that KS and AT domains (and the DH domain, when present) form a single functional unit in a PKS module. This model is mechanistically appealing because the KS and AT domains do not interact directly with each other in the catalytic cycle and therefore could be fixed in position relative to one another. It is also supported by the limited proteolytic studies of Wakil and co-workers on the vertebrate fatty acid synthase (*24*).

When a recombinant ACP is added to the reaction mixture, both the KS-AT-KR^0 and KS-AT fragments are capable of catalyzing efficient acyl transfer and condensation reactions. Furthermore, neither the large interdomain linker nor a simple covalent linkage between either of these multidomain proteins and ACP appears to be required for chain elongation. This finding, which is in contrast to the vertebrate fatty acid synthase (*25*), might suggest that interactions between the KS and ACP are not required for chain elongation. However, because methylmalonyl-CoA (which also has a phosphopantetheinyl moiety) is unable to substitute for methylmalonyl-ACP in the condensation reaction (data not shown), the results of this study suggest that the KS and ACP domains of a module engage via noncovalent protein−protein interactions between specific surface residues rather than linkers. Similar interactions between ACP and KS domains from adjacent modules have also been detected (*13*).

From a protein-engineering standpoint, we have identified two new interdomain fusion junctions, corresponding to

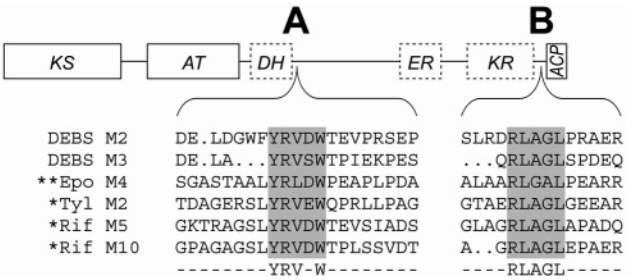


FIGURE 6: (A) Portions of the protein sequence from AT-KR, DH-KR (denoted by a single asterisk), or DH-ER (denoted by a double asterisk) linkers, illustrating the location of the conserved YRVXW fusion junction for a selection of PKS modules (Epo, epothilone synthase; Tyl, tylactone synthase; and Rif, rifamycin synthase). (B) Portions of the KR-ACP linker, illustrating the location of the conserved RLAGL fusion junction for a selection of PKS modules. Linker sequences were obtained from PKSDB, a database of modular polyketide synthases (www.nii.res.in/pksdb.html).

conserved YRVXW and RLAGL downstream of most AT and KR domains, respectively. Interestingly, the YRVXW sequence is also found in other PKSs such as epothilone and rifamycin synthase (Figure 6). Utilizing the YRVXW junction sequence, we have successfully expressed recombinant KS-AT didomain from DEBS module 3 (Figure 5A). In the presence of methylmalonyl- or malonyl-ACP, this recombinant KS-AT protein shows condensation activity similar to that of trypsin-proteolyzed fragments. However, the efficiency of the condensation reaction appears to vary depending on which ACP is used. Therefore, for future construction of hybrid PKS modules, the YRVXW junction could be used to replace KS-AT didomains between different PKS modules. Most protein-engineering experiments thus far have focused on substituting only the AT domain. The resulting hybrid PKSs often synthesize the expected compounds, albeit at substantially lowered yields when compared to corresponding wild-type systems. This may be due to an unstable KS-AT structural unit (*26*) and could be alleviated by preserving the KS-AT interface of individual PKS modules. Similarly, the β-ketoester-modifying enzymes (KR, DH, and/or ER) could be exchanged between modules by engineering protein fusions at the YRVXW and RLAGL sites. It should be noted that the activity of both types of hybrid modules would depend on efficient interactions between the acyl-ACP and heterologous KS, AT, DH, ER, and/or KR domains. In our study, the KS3 shows a preference for ACP2, ACP3, and ACP4 over ACP6. Therefore, the catalytic efficiency of a hybrid module could be optimized by either retaining the endogenous ACP (downstream of the RLAGL site) or by replacing it with a compatible heterologous ACP.

Our results here and elsewhere also demonstrate that specificity of a PKS module for the electrophile (acyl) and nucleophile (malonyl or methylmalonyl) substrates is orthogonally controlled, specificity for the former being dictated by the KS domain and for the latter being under the exclusive control of the AT domain. Thus, it appears that the engineering of hybrid modules is primarily a problem of establishing architecturally and dynamically intact modules using heterologous domains, because the programming of enzyme−substrate interactions is intrinsically modular in nature.

ACKNOWLEDGMENT

We thank Pawan Kumar for plasmid pPK223 and Allis Chien at the Stanford Mass Spectrometry Facility (Stanford University, Stanford, CA) for providing help with mass spectrometric analysis.

REFERENCES

1. Walsh, C. T. (2004) Polyketide and nonribosomal peptide antibiotics: Modularity and versatility, *Science 303*, 1805−1810.
2. Cortes, J., Haydock, S. F., Roberts, G. A., Bevitt, D. J., and Leadlay, P. F. (1990) An unusaually large multifunctional polypeptide in the erythromycin-producing polyketides synthase of *Saccharopolyspora erythraea*, *Nature 348*, 176−178.
3. Donadio, S., Staver, M. J., McAlpine, J. B., Swanson, S. J., and Katz, L. (1991) Modular organization of genes required for complex polyketide biosynthesis, *Science 252*, 675−679.
4. Kumar, P., Khosla, C., and Tang, Y. (2004) Manipulation and analysis of polyketide synthases, *Methods Enzymol. 388*, 269−293.
5. Khosla, C., Gokhale, R. S., Jacobsen, J. R., and Cane, D. E. (1999) Tolerance and specificity of polyketide synthases, *Annu. Rev. Biochem. 68*, 219−253.
6. Tsai, S. C., Miercke, L. J., Krucinski, J., Gokhale, R., Chen, J. C., Foster, P. G., Cane, D. E., Khosla, C., and Stroud, R. M. (2001) Crystal structure of the macrocycle-forming thioesterase domain of the erythromycin polyketide synthase: Versatility from a unique substrate channel, *Proc. Natl. Acad. Sci. U.S.A. 98*, 14808−14813.
7. Tsai, S. C., Lu, H., Cane, D. E., Khosla, C., and Stroud, R. M. (2002) Insights into channel architecture and substrate specificity from crystal structures of two macrocycle-forming thioesterases of modular polyketide synthases, *Biochemistry 41*, 12598−12606.
8. Broadhurst, R. W., Nietlispach, D., Wheatcroft, M. P., Leadlay, P. F., and Weissman, K. J. (2003) The structure of docking domains in modular polyketide synthases, *Chem. Biol. 10*, 723−731.
9. Wu, N., Kudo, F., Cane, D. E., and Khosla, C. (2000) Analysis of the molecular recognition features of individual modules derived from the erythromycin polyketide synthase, *J. Am. Chem. Soc. 122*, 4847−4852.
10. Gokhale, R. S., Tsuji, S. Y., Cane, D. E., and Khosla, C. (1999) Dissecting and exploiting intermodular communication in polyketide synthases, *Science 284*, 482−485.
11. Walsh, C. T., Gehring, A. M., Weinreb, P. H., Quadri, L. E. N., and Flugel, R. S. (1997) Post-translational modification of polyketide and nonribosomal peptide synthases, *Curr. Opin. Chem. Biol. 1*, 309−315.
12. Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989*) Molecular Cloning: A Laboratory Manual*, 2nd ed., Cold Spring Harbor Laboratory Press, New York.
13. Wu, N., Cane, D. E., and Khosla, C. (2002) Quantitative analysis of the relative contributions of donor acyl carrier proteins, acceptor ketosynthases, and linker regions to intermolecular transfer of intermediates in hybrid polyketide synthases, *Biochemistry 41*, 5056−5066.
14. Wu, N., Tsuji, S. Y., Cane, D. E., and Khosla, C. (2001) Assessing the balance between protein−protein interactions and enzyme−substrate interactions in the channeling of intermediates between polyketide synthase modules, *J. Am. Chem. Soc. 123*, 6465−6474.
15. Marsden, A. F., Caffrey, P., Aparicio, J. F., Loughran, M. S., Staunton, J., and Leadlay, P. F. (1994) Stereospecific acyl transfers on the erythromycin-producing polyketide synthase, *Science 263*, 378−380.
16. Pan, H., Tsai, S., Meadows, E. S., Miercke, L. J., Keatinge-Clay, A. T., O'Connell, J., Khosla, C., and Stroud, R. M. (2002) Crystal structure of the priming β-ketosynthase from the R1128 polyketide biosynthetic pathway, *Structure 10*, 1559−1568.
17. Keatinge-Clay, A. T., Maltby, D. A., Medzihradszky, K. F., Khosla, C., and Stroud, R. M. (2004) An antibiotic factory caught in action, *Nature Struct. Mol. Biol. 11*, 888−893.
18. Keatinge-Clay, A. T., Shelat, A. A., Savage, D. F., Tsai, S. C., Miercke, L. J., O'Connell, J. D. III, Khosla, C., and Stroud, R. M. (2003) Catalysis, specificity, and ACP docking site of *Streptomyces coelicolor* malonyl-CoA:ACP transacylase, *Structure 11*, 147−154.
19. Crump, M. P., Crosby, J., Dempsey, C. E., Parkinson, J. A., Murray, M., Hopwood, D. A., and Simpson, T. J. (1997) Solution structure of the actinorhodin polyketide synthase acyl carrier protein from *Streptomyces coelicolor* A3(2), *Biochemistry 36*, 6000−6008.
20. Li, Q., Khosla, C., Puglisi, J. D., and Liu, C. W. (2003) Solution structure and backbone dynamics of the holo form of the frenolicin acyl carrier protein, *Biochemistry 42*, 4648−4657.
21. Findlow, S. C., Winsor, C., Simpson, T. J., Crosby, J., and Crump, M. P. (2003) Solution structure and dynamics of oxytetracycline polyketide synthase acyl carrier protein from *Streptomyces rimosus*. *Biochemistry 42*, 8423−8433.
22. Banerjee, R. V., Johnston, N. L., Sobeski, J. K., Datta, P., and Matthews, R. G. (1989) Cloning and sequence analysis of the *Escherichia coli* metH gene encoding cobalamin-dependent methionine synthase and isolation of a tryptic fragment containing the cobalamin-binding domain, *J. Biol. Chem. 264*, 13888−13895.
23. Aparicio, J. F., Caffrey, P., Marsden, A. F., Staunton, J., and Leadlay, P. F. (1994) Limited proteolysis and active-site studies of the first multienzyme component of the erythromycin-producing polyketide synthase, *J. Biol. Chem. 269*, 8524−8528.
24. Mattick, J. S., Tsukamoto, Y., Nickless, J., and Wakil, S. J. (1983) The architecture of the animal fatty acid synthetase. I. Proteolytic dissection and peptide mapping, *J. Biol. Chem. 258*, 15291−15299.
25. Chirala, S. S., Jayakumar, A., Gu, Z. W., and Wakil, S. J. (2001) Human fatty acid synthase: Role of interdomain in the formation of catalytically active synthase dimer, *Proc. Natl. Acad. Sci. U.S.A. 98*, 3104−3108.
26. Hans, M., Hornung, A., Dziarnowski, A., Cane, D. E., and Khosla, C. (2003) Mechanistic analysis of acyl transferase domain exchange in polyketide synthase modules, *J. Am. Chem. Soc. 125*, 5366−5374.

BI048418N