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Functional Modular Dissection of DEBS1-TE Changes Triketide Lactone Ratios and Provides Insight into Acyl Group Loading, Hydrolysis, and ACP Transfer

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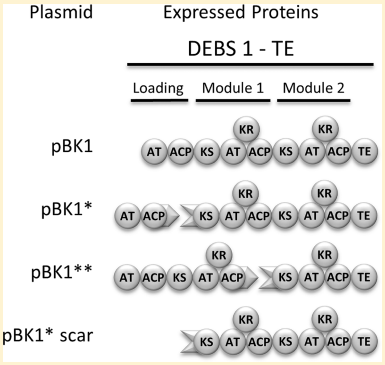
Supporting Information

ABSTRACT: The DEBS1-TE fusion protein is comprised of the loading module, the first two extension modules, and the terminal TE domain of the *Saccharopolyspora erythraea* 6-deoxyerythronolide B synthase. DEBS1-TE produces triketide lactones that differ on the basis of the starter unit selected by the loading module. Typical fermentations with plasmid-based expression of DEBS1-TE produce a 6:1 ratio of propionate to isobutyrate-derived triketide lactones. Functional dissection of the loading module from the remainder of DEBS1-TE results in 50% lower titers of triketide lactone and a dramatic shift in the production to a 1:4 ratio of propionate to isobutyrate-derived products. A series of radiolabeling studies of the loading module has shown that transfer from the AT to the ACP occurs much faster for propionate than for isobutyrate. However, the equilibrium occupancy of the AT favors isobutyrate such that propionate is outcompeted for ACP occupancy. Thus, propionyl-ACP is the kinetic product, while isobutyryl-ACP is the thermodynamic product. A slowed transfer from the loading domain ACP to first-extension module KS due to functional dissection of DEBS1-TE allows this isobutyryl-ACP-favored equilibrium to be realized and likely accounts for the observed shift in triketide lactone products.



The first modular type I PKS to be thoroughly characterized was the 6-deoxyerythronolide B (DEBS) PKS from *Saccharopolyspora erythraea*, which produces the macrocyclic lactone core of the antibiotic erythromycin A.[1−4] Unlike many other acyl transferase (AT) domains that typically display strict substrate specificity,[5,6] the loading module ATs (AT_L) from the DEBS and avermectin systems have displayed relaxed substrate specificity, capable of incorporating a variety of starter units.[7−10] While propionate is the natural starter unit for erythromycin biosynthesis, DEBS AT_L has been shown to load starter units such as acetyl, butyryl, isobutyryl, cyclopropionate, and cyclobutyryl. Further, DEBS has been shown to process these alternate starter units into their final corresponding products, albeit with much lower levels.[6−9,11] DEBS AT_L lies at the N-terminus of DEBS1, which consists of the loading module and the first two extension modules. Previously reported work has shown that in vivo expression of DEBS1 covalently fused at its C-terminus to the TE domain of DEBS3 in an appropriate host such as *S. erythraea*, *Streptomyces coelicolor*, or *Streptomyces venezuelae* results in the production of triketide lactone (TKL) products.[8,12,13] In all cases, a TKL generated from a propionate starter unit predominates (Figure 1).

A series of type I PKS modules may consist of a single polypeptide or several separate polypeptides. Modules on a single chain are connected by a short intraprotein linker, typically ∼10−30 amino acids.[14,15] Interactions between modules on distinct chains are facilitated by docking domains found at the C- and N-termini. These docking domains have been shown to be essential in promoting the appropriate protein−protein and thus module−module interactions required for the polyketide biosynthetic processes.[16−20] We have previously reported that the multimodular polypeptide PikAI, from the pikromycin/methymycin (Pik) PKS, can be functionally dissected into two separate polypeptides while retaining approximately 50% production levels compared to that of the intact polypeptide.[21] This dissection was accomplished by replacing the natural intraprotein linker at either the loading module−module 1 junction or the module 1−module 2 junction with a docking domain pair from the phoslactomycin (Plm) PKS at the respective C- and N-termini.

On the basis of the similarities in the modular organization of DEBS1 and PikAI, we sought to expand on the successful results from the separation of PikAI by determining if separating DEBS1-TE at the module−module junctions would again result in an efficient dissected PKS system. Separation of this multimodular PKS polypeptide was accomplished by replacing the natural intraprotein linkers in DEBS1-TE with heterologous N- and C-terminal docking domains from the phoslactomycin PKS and the pikromycin PKS. The Plm1−Plm2 docking domain pair was selected because of its previous success in the pikromycin PKS dissection. The PikA3−PikA4 docking domain pair, for which a crystal structure (Protein Data Bank entry 3F5H) was

Received: June 20, 2012
Revised: October 15, 2012
Published: November 1, 2012

   dx.doi.org/10.1021/bi300830q | *Biochemistry* 2012, 51, 9333−9341

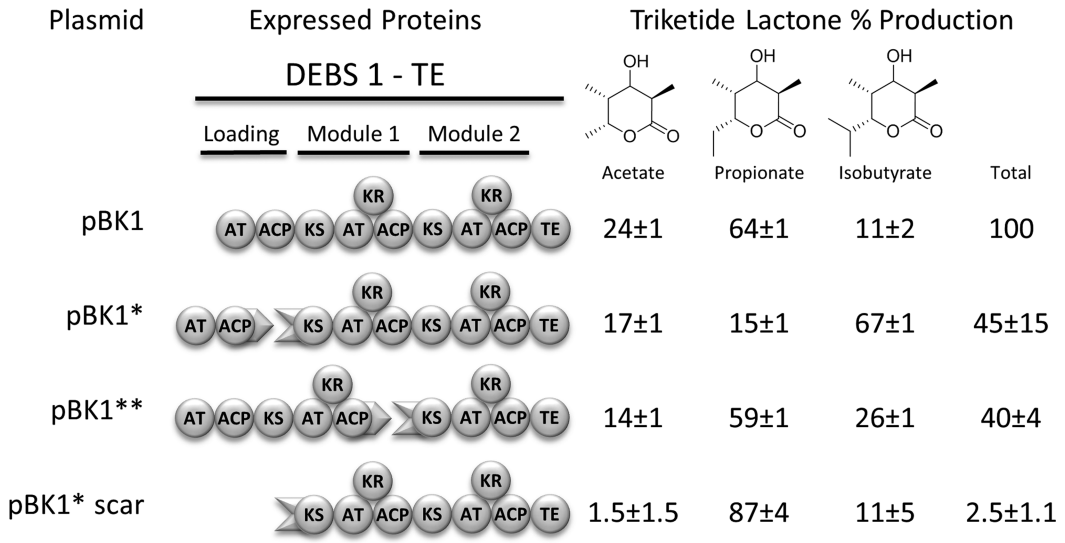


Figure 1. Module organization and triketide lactone production levels for DEBS1-TE expression constructs pBK1, pBK1*, pBK1**, and pBK1* scar expressed in BB138. Docking domains are depicted as either an outward point or an inward crevice. Percentage values for each of the observed acetate-, propionate-, or isobutyrate-derived TKL products were determined by integration of GC peaks from the extracted ion chromatogram of each product. Total TKL percentage values for fermentations of BB138 carrying pBK1*, PBK1**, and pBK1* scar are relative values compared to that produced by intact DEBS1-TE (pBK1).

recently published, was selected on the basis of intriguing binding data showing that this pair exhibited the highest binding affinity for one another when compared to the affinities of other DEBS and Pik docking domain pairs.[22] Insertion of these docking domains was accomplished by generating variants of expression construct pBK1,[12] which expresses DEBS1-TE and produces acetate-, propionate-, and isobutyrate-derived triketide lactones when expressed in *S. venezuelae*.

Here we report the functional dissection of the multimodular polypeptide DEBS1-TE, which has provided an additional example of the utility of matched docking domain pairs to separate linked PKS polypeptides at the module−module junctions. However, the separation of DEBS1-TE at the loading module−module 1 junction resulted in an unexpected shift favoring the utilization of isobutyrate over propionate as the preferred starter unit. This observation along with kinetic analysis has revealed insights into the different rates of acyl group loading and offloading from the AT_L domain, as well as rates of transfer from AT_L to ACP_L for different potential starter units.

MATERIALS AND METHODS

Plasmids, Strains, and Culture Conditions. All constructed plasmids were derived from pBK1, a DEBS1-TE expression plasmid,[12] by a series of polymerase chain reactions (PCRs) and subsequent PCR-targeted recombination crossovers. All plasmid manipulations were completed in *Escherichia coli* hosts before final transformation into *S. venezuelae*. Transformants were cultivated on LB/agar medium and plasmids isolated from LB liquid medium, both supplemented with appropriate antibiotics. Generated variants of pBK1 were transformed into BB138 or AX912 by established protocols[23] onto R2YE agar plates supplemented with the appropriate antibiotics. Transformants were subsequently grown on SPA agar plates (50 mg of thiostrepton/L). SCM liquid medium was used for fermentation and propagation of *S. venezuelae* strains.

DNA Manipulation. Previously reported construct pJY2[21] was used as a template for the amplification of PCR products containing the encoding region for the apramycin resistance marker (flanked with Bstz71I restriction enzyme sites for later removal) and the Plm DD pair along with the needed 39 nucleotides complementary to the desired point of recombination between the linked ACP and KS domains of DEBS1. PCR products for the exchange of DNA sequences encoding the intraprotein linkers between LD module 1 and modules 1 and 2 of DEBS1 with that encoding the Plm docking domain pair were amplified with primer pairs DEBS LD sep For (5′-CACCCGACTCCGCGTGCGCTCGCGGAAGCACTCGCGGCG***CTGGGGCACCTCGATTTCGG***-3′) and DEBS LD sep Rev (5′-GGGCAGCCGGCAGGCCATCGCGACGACCGCGACCGGTTC***GTGGTGCTTCGTCTCGGCGT***-3′) and DEBS M1-M2 sep For (5′-GACGTGCGGACGCTGGCGGCGCACCTGGCCGCCGAACTC***CTGGGGCACCTCGATTTCGG***-3′) and DEBS M1-M2 sep Rev (5′-GGGCAGCCGGCACGCCATGCCGACGATCGCGATCGGCTCG***GTGGTGCTTCGTCTCGGCGT***-3′), respectively. The bold and italic primer sequences represent annealing sites on the template pJY2 and the remaining 5′ region of the needed complementary 39 nucleotides of the DEBS1 region for recombination, respectively. PCR products were introduced into pBK1 by homologous recombination via established protocols.[24] Recombinant pBK1 plasmids were isolated from *E. coli* using the GeneJET Plasmid Miniprep Kit (Fermentas) and confirmed by sequencing. Recombinant plasmids were treated with Bstz71I to remove the apramycin marker, ligated overnight at 12 °C with T4 DNA ligase (New England Biolabs), and transformed into Mach1 *E. coli* chemically competent cells (Invitrogen). Single colonies were screened for both (+) Amp and (−) Apra, yielding pBK1* and pBK1**, respectively.

Plasmid pBK1* scar, which expresses only modules 1 and 2 of DEBS1, was generated from pBK1* by the PCR-targeted *Streptomyces* gene replacement method.[24] For this, the DNA encoding the polypeptide containing the loading domain and the N-terminal PLM docking domain in pBK1* was replaced with the kanamycin resistance marker. This marker was amplified from pKD13[24] with primers DEBS LD scar For

dx.doi.org/10.1021/bi300830q | *Biochemistry* 2012, 51, 9333−9341

(5′-GGGCAGCCGGCACGCCATGCCGACGATCGCGAT-CGGCTCG***ATTCCGGGGATCCGTCGACC***-3′) and DEBS LD scar Rev (5′-GCGACGGGAACAGGGGTGGTGGCAG-AGGATGTATACTCA***TGTAGGCTGGAGCTGCTTC***-3′) (bold and italic sequences represent annealing sites for the antibiotic marker with the remaining 5′ regions representing recombination crossover sites). Successful replacement in pBK1* was followed by FLP-mediated excision of the marker region generating pBK1* scar, which encodes the expression of DEBS1 module 1 with the C-terminal docking domain of Plm2−3 at the N-terminus.

Expression construct pJY105, which expresses DEBS1-TE with the PikA3-A4 DD pair in place of the intrapolypeptide linker between module 1 and module 2, was generated in a manner analogous to that of the complementation constructs mentioned above. The PikAIII-PikAIV docking domain was amplified with primers PikA3A4 DD For (5′-CACGAGGCG-TACCTCGCAC-3′) and PikA3A4DD Rev (5′-CCGGATTC-CGCCCGCGAA-3′) from template pDHS0031 (D. H. Sherman, unpublished observations). PCR products were purified and cloned with the TOPO GW cloning kit (Invitrogen), yielding pJY101. The apramycin *aac(3)IV* resistance marker was amplified from pIJ773,[24] using primers PikA3A4DD apra rec For (5′-GCCCTGATCCGGATGGCT-CTCGGCCCCCGTAACACCTGAGTA*TAC**GGTTCATG-TGCAGCTCCATC**-3′) and PikA3A4 apra rec Rev (5′-TGCGCGGGATGCGCGGGGTGCGGCGCGTGGGGCAG-GACCGTA*TAC**TGTAGGCTGGAGCTGCTTC**-3′) (bold sequences represent annealing sites for the antibiotic marker, and the asterisk indicates the locations of Bstz17I restriction sites introduced for later removal of this marker). The resulting PCR product was recombined as before, via established procedures. The resulting recombinant plasmid pJY102 was isolated with the GeneJET Plasmid Miniprep Kit (Fermentas) and confirmed by sequencing. Construct pJY102 was used as a template to generate a PCR product with the needed 39 nucleotides complementary to the desired point of recombination between linked ACP module 1 and the KS of module 2 of DEBS1. PCR products for the exchange of DNA sequences encoding the intramodular linkers between modules 1 and 2 for that coding for the PikAIII/PikAIV DD pair were amplified with the primer pair of DEBS A3-A4 rec For (5′-CACCCG-ACTCCGCGTGCGCTCGCGGAAGCACTCGCGGCG**CA-CGAGGCGTACCTCGCACC**-3′) and DEBS A3-A4 rec Rev (5′-GGGCAGCCGGCAGGCCATCGCGACGACCGCGAC-CGGTTCT**CCTGCCGACGGTCGGCCCGG**-3′) (bold sequences represent annealing sites on template pJY102, and remaining 5′ regions represent the complementary 39 nucleotides of the DEBS1 region for recombination). PCR products were gel purified and introduced into pBK1 by homologous recombination via established protocols. Recombinant pBK1 plasmids were isolated as before, treated with Bstz71I to remove the apramycin marker, ligated overnight at 12 °C, and transformed into *E. coli*. Single colonies were screened for both (+) Amp and (−) Apra, yielding pJY105.

Triketide Lactone Production Analysis. Triketide lactone production was analyzed by three triplicate sets of fermentations of *S. venezuelae* PikAI deletion strain BB138 transformed with the appropriate DEBS1-TE expression plasmids (pBK1, pBK1*, pBK1**, pBK1* scar, and pJY105). Fermentations were conducted in 10 mL of SCM medium supplemented with 50 mg/L thiostrepton at 30 °C with agitation (220 rpm) for 72 h. After fermentation, the entire culture medium was extracted three times with 10 mL of ethyl acetate and dried. Extracts were treated with 200 μL of Trisil-Z (Pierce-Thermo Scientific) derivatization reagent for 20 min at 60 °C to form their trimethyl silyl ester (TMS) derivative. Reactions were quenched with 1 mL of water and mixtures allowed to cool to room temperature. The reaction mixture was extracted three times with 1 mL of $CHCl_3$. The collected $CHCl_3$ extracts were concentrated to a final volume of 0.5 mL. Derivatized extracts of *S. venezuelae* BB138 carrying the appropriate expression plasmids were analyzed by GC−MS.

GC−MS Detection of Triketide Lactones. Analysis was conducted using an Agilent Technologies 6890N GC system along with an Agilent Technologies 5973 mass selective detector. Triketides were identified by characteristic fragment ions at *m/e* 73, 115, 130, and M^+ (depending on the starter unit). TKLs derived from acetate, propionate, and isobutyrate were identified by their expected retention times and *m/z* values of 157 (7.9 min), 171 (8.3 min), and 185 (8.5 min) respectively, corresponding to published data for the expression of pBK1 in *S. venezuelae*.[12] In addition to each individual M^+ ion, TKLs were also identified by characteristic fragment ions with *m/z* values of 73 and 115.

DEBS AT_L-ACP_L Expression and Purification. The DEBS AT_L-ACP_L didomain was overexpressed as a C-terminal His_6-tagged protein and purified from *E. coli* using previously reported expression plasmid pBK12.[25] Expression was conducted in either BL21 DE3 chemically competent cells (Invitrogen) or BAP1 cells. BAP1 *E. coli* cells are a derivative of BL21 DE3 cells, which contains a chromosomally integrated copy of the *sfp* gene encoding the requisite 4′-phosphopantetheinyl transferase, required for posttranslational modification of the ACP domain to the holo form. BAP1 *E. coli* cells were provided as a gift from C. Khosla's laboratory (Stanford University, Stanford, CA).

LB medium (1 L) supplemented with ampicillin (100 μg/mL) was inoculated with 5% (v/v) of an overnight seed culture harboring pBK12 and grown at 37 °C to an OD_{600} of 0.6. The medium was allowed to cool to room temperature, and expression was induced by 0.5 mM isopropyl thio-β-D-galactoside (IPTG). Cultures were grown for an additional 16 h at room temperature. Cells were harvested by centrifugation and stored at −80 °C. BugBuster Protein Extraction Reagent (Novagen) and sonication were used for cell lysis. The His_6-tagged protein was purified according to the native conditions protocol outlined in Qiagen's QiAexpressionist handbook. The target protein was further purified to homogeneity by size exclusion chromatography using an AKTA FPLC System (GE Healthcare, formally Amersham Biosciences) equipped with a Superdex GL 200 sizing column and a UV detector set at 280 nm. Fractions containing the protein were confirmed by an sodium dodecyl sulfate−polyacrylamide gel electrophoresis band between 50 and 75 kDa; DEBS AT_L-ACP_L has a molecular mass of approximately 59 kDa (see Figure S1 of the Supporting Information). Fractions were concentrated with Amicon Ultra Centrifuge columns (Millipore) and stored at −80 °C.

LC−MS Analysis of DEBS AT_L-ACP_L. Both apo and holo forms of DEBS AT_L-ACP_L were analyzed by LC−MS in positive mode on a Thermo Scientific LTQ-Orbitrap Discovery high-resolution mass spectrometer with a dedicated Accela HPLC system using a Discovery BioWide Pore C5 (5 cm × 2.1 mm, 3 μm, Supelco) column at a flow rate of 0.3 mL/min. The following solvent gradient between buffer A (0.05% TFA in

water) and buffer B (0.05% TFA in acetonitrile) was used in the analysis (time, % buffer B): 0 min, 1%; 5 min, 1%; 25 min, 99%; 28 min, 99%; 30 min, 1%; and 33 min, 1%. MS data were deconvoluted with MagTran 1.0 Beta 8.[26]

Radioactive Labeling of DEBS AT_L-ACP_L. Heterologously expressed apo and holo AT_L-ACP_L was used for ^{14}C radiolabeling experiments involving [^{14}C]propionyl and [^{14}C]-isobutyryl-CoA substrates purchased from American Radiolabeled Chemicals. Both materials were supplied with specific activities of 50 μCi/μmol. AT_L-ACP_L (4 μM) was incubated under four different sets of conditions: [^{14}C]propionyl-CoA (200 μM, 25 μCi/μmol), [^{14}C]isobutyryl-CoA (200 μM, 25 μCi/μmol), [^{14}C]propionyl-CoA (100 μM, 50 μCi/μmol) with [^{12}C]isobutyryl-CoA (100 μM), and [^{14}C]isobutyryl-CoA (100 μM, 50 μCi/μmol) with [^{12}C]propionyl-CoA (100 μM) with all assays in 100 mM NaH_2PO_4, 1 mM TCEP, 1 mM EDTA, and 20% glycerol (pH 7.4). The extent of protein labeling was determined by removing 25 μL aliquots at 0, 5, 7.5, 15, and 30 min. Protein samples were precipitated with ice-cold 10% TCA (400 μL) supplemented with 40 μg of BSA, acting as a carrier protein. Samples were centrifuged at 14500 rpm (14000g) for 10 min, washed with another portion of 10% TCA (200 μL), and centrifuged for an additional 5 min. Precipitated protein samples were resuspended in a solution of 2% SDS and 20 mM NaOH (50 μL) for liquid scintillation counting.

Measurement of Acyl-CoA Loading and Hydrolysis. By monitoring the rates of protein-dependent free CoA formation with different acyl-CoA substrates (acetyl, propionyl, and isobutyryl), we determined k_{cat} and K_m values for the apo form of DEBS AT_L-ACP_L. The formation of free CoA was monitored via a fluorescence assay using the commercially available Thioglo reagent from Covalent Associates Inc. (Corvallis, OR). Thioglo is a commercially available reagent that derivatizes all free thiol-containing compounds and fluoresces when excited at 379 nm. k_{cat} and K_m values were determined in three duplicate sets; values were averaged, and the standard error was calculated. A mixture containing 1.5 μM protein was incubated at room temperature with varying concentrations of each individual acyl-CoA substrate (acetyl, propionyl, butyryl, and isobutyryl) in a buffer containing 100 mM NaH_2PO_4, 0.2 mM TCEP, 1 mM EDTA, and 20% glycerol (pH 7.4). Aliquots (25 μL) were removed after incubation for 30 min and placed in individual wells of a 96-well plate preincubated on ice. The reactions were quenched by the addition of 75 μL of DMSO. To each aliquot was added 100 μL of 200 μM Thioglo in DMSO, and the plate was allowed to incubate at room temperature with gentle agitation for 25 min. A standard curve was generated by preparing 0, 25, 50, 100, 200, and 400 μM free CoA in the same assay buffer followed by the same workup procedure that is described above. Excitation at 379 nm and emission monitoring between 400 and 540 nm were completed with a Gemini EM microplate spectrofluorometer (Molecular Devices), equipped with Softmax Pro.

Calculation of Thermodynamic Parameters. The equilibrium constant for the acylation of AT_L (K_{acyl}) was calculated as the ratio of labeled apoprotein to unlabeled apoprotein after incubation for 10 min. The rate of formation of free CoA (k_{cat}) depends on the rates of acylation (k_A) and hydrolysis (k_H) according to eq 1.

$$k_{cat} = \frac{k_A k_H}{k_A + k_H} \quad (1)$$

The AT_L acylation equilibrium depends on k_A and k_H according to eq 2.

$$K_{acyl} = \frac{k_A}{k_H} \quad (2)$$

The acylation and hydrolysis rates were calculated from eqs 1 and 2 using the experimentally determined k_{cat} and K_{acyl}. See Figure 2 for a scheme depicting k_A and k_H as used in eqs 1 and 2. To determine the equilibrium constant for the transfer of the starter unit from AT_L to ACP_L (K_{trans}), we calculated the levels of different labeled holo AT_L-ACP_L species. The possibility of [^{14}C]carboxylates being bound to AT_L and ACP_L simultaneously complicated this calculation and led to eq 3 for the concentration of unlabeled protein ([E]) based on the total protein ([T]) and bound [^{14}C]carboxylates ([^{14}C]). Once [E] is known, calculations for concentrations of the labeled holo species are trivial and K_{trans} is determined by the ratio of labeled ACP_L to labeled AT_L.

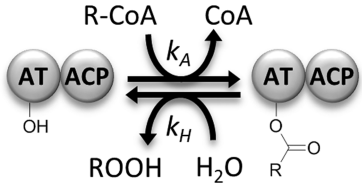


Figure 2. Schematic of the acylation and hydrolysis model of the apo DEBS1-TE loading domain. Multiple turnovers lead to detectable free CoA accumulation in in vitro experiments. Equations 1 and 2 are based on this model.

$$[E] = \frac{[T](2 + K_{acyl}^{-1}) - [^{14}C](1 + K_{acyl}^{-1})}{2 + K_{acyl} + K_{acyl}^{-1}} \quad (3)$$

RESULTS

Triketide Lactone Production by pBK1-Based Expression Constructs. The pBK1 construct expresses DEBS1-TE as an intact polypeptide. To examine the effect of separating this type 1 PKS at the module junctions, we created the pBK1* and pBK1** constructs that express DEBS1-TE as two separate proteins, with docking domains engineered at the module junctions. In pBK1*, a docking domain sits at the C-terminus of the loading module, and a corresponding docking domain sits at the N-terminus of module 1. In pBK1**, this docking domain pair sits between modules 1 and 2. The total levels of production of TKLs resulting from expression of dissected DEBS1-TE from pBK1* and pBK1** were 45 ± 15 and 40 ± 4%, respectively, compared to the level of expression of nondissected DEBS1-TE from pBK1 in strain BB138 (Figure 1). This approximate halving of production is comparable to levels observed in the dissected pikromycin system previously described.[21] However, expression of DEBS1-TE dissected between the loading module and module 1 (construct pBK1*) resulted in an unexpected shift in starter unit utilization toward isobutyrate. Previous reports have shown substrate flexibility in the DEBS loading module, but the predominate starter unit has always remained propionate.[7,8,27,28] Published evidence has also shown that a strain of *S. erythraea* from which DEBS AT_L-ACP_L has been deleted still retains the ability to produce erythromycin, albeit at a much diminished level.[29] This evidence of direct KS domain

dx.doi.org/10.1021/bi300830q | *Biochemistry* 2012, 51, 9333−9341

loading made it essential to determine if the observed shift in TKL ratios arose through the change in the expression of DEBS AT_L-ACP_L as a stand-alone protein and not simply the direct loading of DEBS KS1 with isobutyrate. This was achieved by constructing the AT_L-ACP_L deletion variant pBK1* scar that expresses the C-terminal Plm docking domain fused to the N-terminus of the KS of extension module 1. The level of production by BB138/pBK1* scar was approximately 2% of that observed from expression of intact DEBS1-TE, 90% of which was derived from propionate (Figure 1).

Impact of Utilizing the PikAIII-PikAIV Docking Domain Pair To Dissect DEBS1-TE. In addition to the Plm docking domain pair, we examined the effect of dissecting DEBS1-TE with a docking domain pair from the Pik PKS. Construct pJY105 was designed in a manner analogous to that of pBK1*. In this case, the docking domains from the PikAIII C-terminus and PikAIV N-terminus were used to separate the loading module from module 1 in DEBS1-TE. Initial expression of this construct in PikAI deletion strain BB138 generated no detectable TKL products. Because of the possibility that interactions between the inserted Pik docking domains and the naturally expressed PikAIII/PikAIV docking domains could inhibit TKL production, pJY105 was alternatively expressed in a PikAIII-PikAIV deletion strain of *S. venezuelae*, YJ004.[30] The expression of pJY105 in YJ004 resulted in trace levels of TKL production, which were approximately 1% of that in the wild-type control experiment (data not shown).

Heterologous Expression of DEBS AT_L-ACP_L. A series of in vitro experiments were conducted with just DEBS AT_L-ACP_L. These experiments differ from in vivo studies, in which the remaining modules of DEBS1-TE are present as a separate protein but were designed to probe possible reasons for the shift in TKL starter units (Figure 1) with separation of the loading domain, and a slower transfer to module 1.

DEBS AT_L-ACP_L was purified as a C-terminally His_6-tagged protein from *E. coli* harboring the previously described expression construct, pBK12.[25] A two-step purification of the protein in either the apo or the holo form was accomplished by Ni-NTA resin and subsequent size exclusion chromatography (see Figure S1 of the Supporting Information). Purified holo DEBS AT_L-ACP_L was analyzed by LC−MS to determine the level of phosphopantetheinylation (see Figure S2 of the Supporting Information). As a control, the apoprotein was also analyzed. A single protein species eluting at 17.2 min was observed in the total ion chromatogram (TIC) that has a mass of 59197 Da, consistent with the predicted mass of the holoprotein. Additionally, the observed mass is 340 Da larger than the observed mass for the apoprotein. The analysis revealed that holo DEBS AT_L-ACP_L purified from BAP1 cells did not contain detectable levels of the apoprotein.

Kinetic Analysis of DEBS AT_L-ACP_L with Varying Acyl-CoA Substrates. Recent work has shown ATs have significant hydrolytic activity, the rate of which depends on the acyl-CoA substrate.[31] Seeking to extend this work and provide insight into the TKL ratio shift, we examined the in vitro hydrolysis of CoA substrates by DEBS AT_L. Kinetic parameters (k_{cat} and K_M) for the apo form of DEBS AT_L-ACP_L were determined by monitoring the protein-dependent formation of free CoA from acetyl-, propionyl-, and isobutyryl-CoA. Determined k_{cat} and K_M values with each substrate are summarized in Table 1. The approximately equivalent catalytic efficiency of DEBS AT_L-ACP_L for the processing of propionyl- and isobutyryl-CoA, as determined by k_{cat}/K_M values of 4.2 and 4.4 mM^{-1} s^{-1}, respectively, does not reflect any significant difference in hydrolytic rates.

Table 1. Kinetic Parameters for Apo DEBS AT_L-ACP_L

substrate	k_{cat} (min^{-1})	K_M (μM)	k_{cat}/K_M (mM^{-1} min^{-1})
acetyl-CoA	1.3 ± 0.1	812 ± 102	1.6
propionyl-CoA	1.5 ± 0.1	360 ± 54	4.2
isobutyryl-CoA	2.1 ± 0.1	486 ± 92	4.4

Radioactive Acylation of DEBS AT_L-ACP_L. To examine the acyl occupancy of the domains within DEBS AT_L-ACP_L, a series of in vitro radioactive labeling experiments involving [^{14}C]propionyl- and [^{14}C]isobutyryl-CoA was conducted with recombinant protein. The goal of this work was to determine the relative equilibrium levels of acylation by different starter units and how these levels are approached with and without competitive substrates. The extent of ^{14}C labeling for the apoprotein was used to examine AT_L domain acylation without the complication of the AT-ACP transfer step, while experiments with the holoprotein provided information about the transfer of acyl groups from the AT_L domain to the ACP_L domain.

In noncompetitive experiments, [^{14}C]acyl-CoA (200 μM, 25 μCi/μmol) was incubated with 4 μM apo or holo DEBS AT_L-ACP_L (Figure 3). Throughout the time course of the experiments with the apoprotein, the level of occupancy was significantly higher in the presence of isobutyryl-CoA (1.6 μM, 40% of the maximum) than in the presence of propionyl-CoA (0.25 μM, 6% of the maximum). Radiolabeling levels of the holoprotein reflect the ability of the [^{14}C]acyl group to occupy either one or both domains of AT_L-ACP_L; this increases the effective maximal concentration of bound acyl groups to 8 μM. Incubation of the holoprotein resulted in increased concentrations of bound [^{14}C]acyl groups relative to that of the apo form for both isobutyrate (3.0 μM, 37% of the maximum) and propionate (1.5 μM, 19% of the maximum). In nearly all noncompetitive experiments, radiolabeling reached equilibrium at the first data point. The exception was holoprotein acylation by isobutyrate, which remained at levels comparable to that of the apoprotein for the initial time point.

In competitive experiments, [^{14}C]propionyl-CoA (100 μM, 50 μCi/μmol) with [^{12}C]isobutyryl-CoA (100 μM) or [^{14}C]isobutyryl-CoA (100 μM, 50 μCi/μmol) with [^{12}C]-propionyl-CoA (100 μM) was incubated with 4 μM apo or holo DEBS AT_L-ACP_L. Results from experiments with the apoprotein reflected those from the noncompetitive assays. Despite the decrease in the level of labeling due to competition from another CoA, the level of radiolabeling of AT_L by isobutyrate (0.8 μM, 20% of the maximum) was again higher than that by propionate (0.3 μM, 8% of the maximum). In contrast, competitive incubation of the holoprotein resulted in a level of labeling by propionate (1.5 μM) that is initially twice that of isobutyrate (0.75 μM). Throughout the 30 min time course, isobutyrate displaces propionate and eventually predominates AT_L-ACP_L occupancy at 1.2 μM bound isobutyrate and 0.75 μM bound propionate after 30 min.

DISCUSSION

The production of TKLs in fermentations of BB138/pBK1* and BB138/pBK1** demonstrates another successful dissection of a multimodular PKS polypeptide by a heterologous docking domain pair. The observed halving of production levels is comparable to dissection of the PikAI polypeptide by the

dx.doi.org/10.1021/bi300830q | *Biochemistry* 2012, 51, 9333−9341

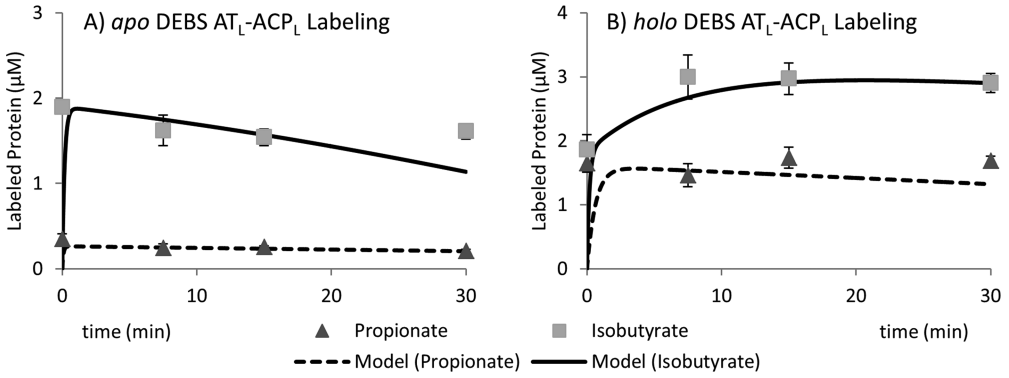


Figure 3. Observed and model predicted levels of DEBS AT$_L$-ACP$_L$ labeling with [^{14}C]propionyl-CoA or [^{14}C]isobutyryl-CoA for (A) the apoprotein and (B) the holoprotein. For each acyl substrate, the discontinuous assay data are shown as points and the data predicted by the continuous model are shown as a line.

same Plm docking domain pair.[21] In contrast, use of the PikAIII-AIV docking domain pair resulted in the near elimination of TKL production, a level of disruption similar to that observed in the absence of any docking domain in the dissected pikromycin system. These results underscore the impact of both the presence and the nature of the docking domains on the ability of a dissected PKS polypeptide to retain production.

The basis for the disparate capabilities of docking domains to provide the necessary protein−protein interactions for the passage of polyketide intermediates is not clearly evident. Data describing PikAIII and PikAIV docking interactions[22] have shown that the binding specificity that docking domains display for one another is dramatically affected by the downstream protein sequence, in this case the entirety of Pik module 6. Additional properties directing a module's interactions with its cognate partner in the biosynthetic process downstream of the KS-AT region have yet to be determined.[19,32] Given these data, it is possible that the C-terminal PikAIII and N-terminal PikAIV docking domains perturb the natural ACP−KS interactions in DEBS1-TE, which play key roles in the proper module−module interactions. Another possible explanation is one in which the N-terminal docking domain that has been fused upstream of the KS domain does not facilitate the adequate expression and proper folding of a functional protein for polyketide production.

The observation that expression of the dissected DEBS1-TE without the cognate loading module results in a 40-fold decrease in the overall level of TKL production indicates that AT$_L$-ACP$_L$ is required for efficient polyketide production. This observation confirms previous published data that mutant strains lacking AT$_L$-ACP$_L$ can still function and produce polyketide products, however with a significant decrease in the overall level of production.[29] The TKLs produced in the absence of the loading module were predominately derived from propionate; this rules out direct loading of the KS as an explanation for the shift to isobutyrate-derived TKLs in BB138/pBK1* fermentations. Furthermore, TKLs produced by DEBS1-TE dissected between module 1 and module 2 were also mostly propionate-derived, indicating the source of the ratio shift lies in the dynamics of separating the loading module from the KS by insertion of a docking domain pair.

One possible explanation for the shift in TKL ratios from the expression of pBK1* is that there are differences in the rates of hydrolysis for different acyl groups bound to DEBS AT$_L$-ACP$_L$. In vitro observations have shown that a variety of PKS proteins, when incubated with acyl-CoA substrates, can result in an increase in the level of free CoA at a rate dependent on the acyl-CoA substrate.[31] In this two-step process, acylation of the AT releases free CoA followed by subsequent hydrolysis and release of the carboxylate. Given these observations, it was hypothesized that when passage of starter units to the KS is slowed by a docking domain pair, the thioesterase activity of DEBS AT$_L$ might act to selectively remove propionate. In this model, propionate bound to DEBS AT$_L$ would be hydrolyzed faster than isobutyrate, resulting in an equilibrium favoring isobutyrate occupancy of ACP$_L$ and its subsequent passage to the KS of module 1. This hypothesis assumes that normal passage from the ACP to the KS active site is relatively fast and the equilibrium of the AT domain is thus not significant in a nondissected polypeptide.

The observed kinetic data for apo DEBS AT$_L$-ACP$_L$ demonstrate a slightly higher rate of formation of free CoA from isobutyryl-CoA than from propionyl-CoA (Table 1), but the equilibrium levels of radiolabeling show this does not lead to higher occupancy by propionate. The observation that apo AT$_L$-ACP$_L$ occupancy is much higher for isobutyrate (40%) than for propionate (6%) is the result of the relative rates of the acylation and subsequent hydrolysis step (Figure 1). The rates of these individual steps were calculated from the overall rate and the equilibrium levels of radiolabeling. The individual rates show that the acylation step is rate-limiting for the formation of free CoA from propionyl-CoA, while hydrolysis is rate-limiting for isobutyryl-CoA (Table 2). Modeling of the radiolabeling assays using the derived rates of acylation and hydrolysis yields a time course consistent with the experimental data (Figure 3). Solely on the basis of the apoprotein data, it is unclear how propionate-derived TKL could predominate even from a nondissected PKS. An examination of the role of ACP$_L$ in the holoprotein is required to understand how propionate starter units are favored by the intact PKS.

Table 2. Calculated Thermodynamic Parameters for Holo DEBS AT$_L$-ACP$_L$

carboxylate	k_A (min^{-1})	k_H (min^{-1})	K_{acyl} (k_A/k_H)	K_{trans}
propionate	1.8	8.9	0.2	6.8
isobutyrate	7.4	2.9	2.5	0.8

Kinetic experiments with holo AT$_L$-ACP$_L$ are complicated by the reversible transfer of the starter unit from AT$_L$ to ACP$_L$. Using the previously calculated AT$_L$ acylation equilibrium and

dx.doi.org/10.1021/bi300830q | *Biochemistry* 2012, 51, 9333−9341

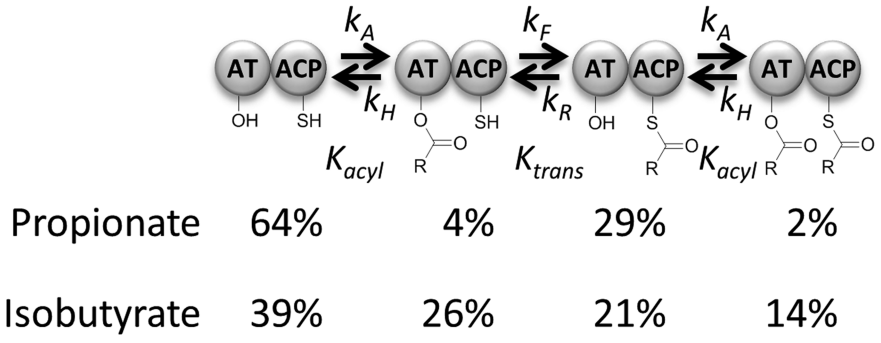


Figure 4. Equilibrium occupancy levels of holo loading module domains. Levels were calculated from radiolabeling data in noncompetitive experiments. Forward and reverse steps are labeled with the corresponding rate and equilibrium constants for AT_L loading and transfer to ACP_L.

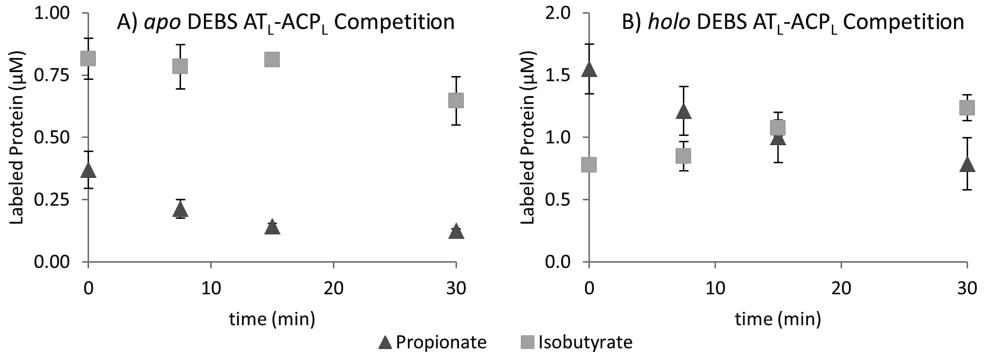


Figure 5. Observed levels of DEBS AT_L-ACP_L labeling with competing acyl-CoA substrates for (A) the apoprotein and (B) the holoprotein. Note the clear exchange of substrates in plot B showing the changeover from kinetically to thermodynamically loaded acyl units.

the level of holoprotein radiolabeling, the occupancy level of ACP_L was calculated. AT_L and ACP_L can simultaneously hold an acyl group; hence, occupancy levels of either domain include doubly acylated protein (Figure 4). Substrates had similar ACP_L occupancies, 35% for isobutyrate [21% + 14% (Figure 4)] and 31% for propionate [29% + 2% (Figure 4)]. The equilibrium constant for the transfer of the starter unit from AT_L to ACP_L (K_{trans}) was calculated from the relative occupancy of AT_L and ACP_L. For propionate, K_{trans} is 6.8, favoring transfer to ACP_L. Isobutyrate transfer has a K_{trans} of 0.8, slightly favoring reverse transfer. Although the absolute transfer rates cannot be quantified from equilibrium data of the holoprotein, isobutyrate transfer appears slower based on the lower initial level of radiolabeling (Figure 3B). This behavior, a faster transfer rate and higher K_{trans} for propionate, is consistent with propionate being the preferred starter unit in nondissected DEB1-TE. The seemingly contradictory observation that the isobutyrate occupancy of ACP_L is comparable to the propionate occupancy despite having a less favorable transfer equilibrium can be attributed to its significantly higher AT_L occupancy.

Competition experiments between isobutyryl-CoA and propionyl-CoA provide insights into how these equilibria and estimates of different rates might affect the levels of ACP_L occupancy during TKL production. Across all radiolabeling experiments, the only times at which the level of propionate labeling is higher than that of isobutyrate are the initial time points of the competitive holoprotein assays (Figure 5). Considering the level of isobutyrate labeling is twice the level of propionate labeling in competitive apoprotein assays, it is clear that propionate dominates the initial time points with the holoprotein because it is more kinetically favored to occupy ACP_L. Over time, however, the holoprotein becomes labeled more by the thermodynamically favored isobutyrate (1.2 μM) than by propionate (0.75 μM). The observed switch from propionate to isobutyrate observed in these competition experiments could be observed in our kinetic models of this competition only when the transfer rate for propionate was set ~100-fold higher than for isobutyrate. The final state of ACP_L occupancy observed in both the model and the experimental data was consistent with those predicted by the equilibrium constants. The intact DEBS1-TE system therefore produces predominately propionate-derived products, because propionate more rapidly occupies ACP_L. However, when a docking domain pair separates the loading module from the extension modules, the ACP_L occupancy may shift toward an isobutyrate-favored equilibrium and TKL production ratios shift accordingly.

Fermentations directed by pBK1** result in a slight shift to isobutyrate-derived TKLs, but not the marked reversal of ratios seen in pBK1* (Figure 1). It seems reasonable that a similar but less pronounced effect of kinetic versus thermodynamic qualities results in this shift.

A growing body of knowledge suggests that an improved understanding of the kinetics of biosynthesis is required to engineer efficient modular PKSs. As is the case in this study, these kinetic parameters can alter the structure of the final product even when yields are not greatly impacted. Previous reports showing DEBS AT_L has relaxed substrate specificity are consistent with our observed AT_L loading kinetics, but the previous studies did not reveal relatively high production levels of the alternate polyketides. Selection of the starter unit by the loading module involves the reversible processes of acylation of AT_L and transfer to ACP_L. By separating modules with a docking domain pair, we changed the biosynthetic process such

dx.doi.org/10.1021/bi300830q | *Biochemistry* 2012, 51, 9333−9341

that the acylation−hydrolysis equilibrium on AT_L, which normally has little effect, now defines the structure of the final product to a greater extent. Whereas intact DEBS1 utilizes the propionate starter unit by kinetically favoring its transfer to ACP_L, the introduction of a downstream rate limit at the KS leads to isobutyrate occupancy at ACP_L because of the upstream equilibrium at AT_L. In this particular instance, extension modules of DEBS1-TE are capable of processing isobutyrate to the final product with reasonable yields. However, in another system, a newly favored starter or extender unit might not be a substrate in later reactions, leading to loss of production. Thus, kinetic and thermodynamic considerations represent another complication for efforts to engineer novel modular and catalytically efficient PKSs.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Detailed DNA manipulation methods. This material is available free of charge via the Internet at http://pubs.acs.org.

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Funding

We gratefully acknowledge funding support from National Institutes of Health Grant R01 GM0876477.

Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

We thank David H. Sherman for helpful conversations concerning DEBS1-TE.

■ ABBREVIATIONS

PKS, polyketide synthase; DEBS, 6-deoxyerythronolide B synthase; TKL, triketide lactone; ACP, acyl carrier protein; AT, acyl transferase; KS, ketosynthase; GC−MS, gas chromatography−mass spectrometry.

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