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Mechanistic Analysis of Acyl Transferase Domain Exchange in Polyketide Synthase Modules

Marcus Hans,‡ Andreas Hornung,‡ Agnieszka Dziarnowski,‡ David E. Cane,† and Chaitan Khosla*,‡

Contribution from the Departments of Chemical Engineering, Chemistry, and Biochemistry, Stanford University, Stanford, California 94306, and Department of Chemistry, Brown University, Providence, Rhode Island 02912

Received December 2, 2002; E-mail: ck@chemeng.stanford.edu

Abstract: Many polyketides are synthesized by a class of multifunctional enzymes called type I modular polyketide synthases (PKSs). Several reports have described the power of predictively altering polyketide structure by replacing individual PKS domains with homologues from other PKSs. For example, numerous erythromycin analogues have been generated by replacing individual methylmalonyl-specific acyl transferase (AT) domains of the 6-deoxyerythronolide B synthase (DEBS) with malonyl-, ethylmalonyl-, or methoxymalonyl-specific domains. However, the construction of hybrid PKS modules often attenuates product formation both kinetically and distributively. The molecular basis for this mechanistic imperfection is not understood. We have systematically analyzed the impact of replacing an AT domain of DEBS on acyl-AT formation, acyl-CoA:HS-NAc acyl transferase activity, acyl-CoA:ACP acyl transferase activity (nucleophile charging), acyl-SNAc:ketosynthase acyl transferase activity (electrophile charging), and β-ketoacyl ACP synthase activity (condensation). As usual, domain junctions were located in interdomain regions flanking the AT domain. Kinetic analysis of hybrid modules containing either malonyl transferase or methylmalonyl transferase domains revealed a 15−20-fold decrease in overall turnover numbers of the hybrid modules as compared to the wild-type module. In contrast, both the activity and the specificity of the heterologous AT domains remained unaffected. Moreover, no defects could be detected in the ability of the heterologous AT domains to catalyze acyl-CoA:ACP acyl transfer. Single turnover studies aimed at directly probing the ketosynthase-catalyzed reaction led to two crucial findings. First, wild-type modules catalyzed chain elongation with comparable efficiency regardless of whether methylmalonyl-ACP or malonyl-ACP were the nucleophilic substrates. Second, chain elongation in all hybrid modules tested was seriously attenuated relative to the wild-type module. Our data suggest that, as currently practiced, the most deleterious impact of AT domain swapping is not on the substrate specificity. Rather, it is due to the impaired ability of the KS and ACP domains in the hybrid module to catalyze chain elongation. Consistent with this proposal, limited proteolysis of wild-type and hybrid modules showed major differences in cleavage patterns, especially in the region between the KR and ACP domains.

Introduction

Polyketides are a structurally diverse group of natural products, which exhibit a broad range of biological activities such as antibiotic, antifungal, immunosuppressive, and anticancer properties.[1] Within the past decade, the genes encoding a number of polyketide biosynthetic pathways have been cloned and sequenced. At the core of each pathway lies a multifunctional polyketide synthase (PKS), which converts metabolically available acyl-CoA precursors into complex polyketide backbones via a stepwise chain building mechanism. All type I and type II PKSs have one or more ketosynthases (KSs), acyl transferases (ATs), and acyl carrier proteins (ACPs). The growing polyketide chain is anchored on the KS via an electrophilic thioester linkage. The AT is responsible for transferring an α-carboxylated nucleophilic extender unit from the corresponding acyl-CoA to the phosphopantetheine arm of the ACP. The acyl-KS and the acyl-ACP then interact to catalyze C−C bond formation, leading to a β-ketoacyl-ACP intermediate that has been extended by two backbone carbon atoms. Upon transfer of this intermediate to the same or different KS, a new round of chain elongation can commence.

A vivid example of a modular PKS is the 6-deoxyerythronolide B synthase (DEBS) from *Saccharopolyspora erythraea*, which produces the polyketide backbone of the antibiotic erythromycin (Figure 1).[2,3] DEBS consists of three large bimodular polypeptides (each >300 kDa) and catalyzes the stepwise condensation of a propionyl-CoA derived primer unit with six methylmalonyl-CoA derived extender units to yield 6-deoxyerythronolide B (6dEB). The modularity of DEBS has

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‡ Brown University.

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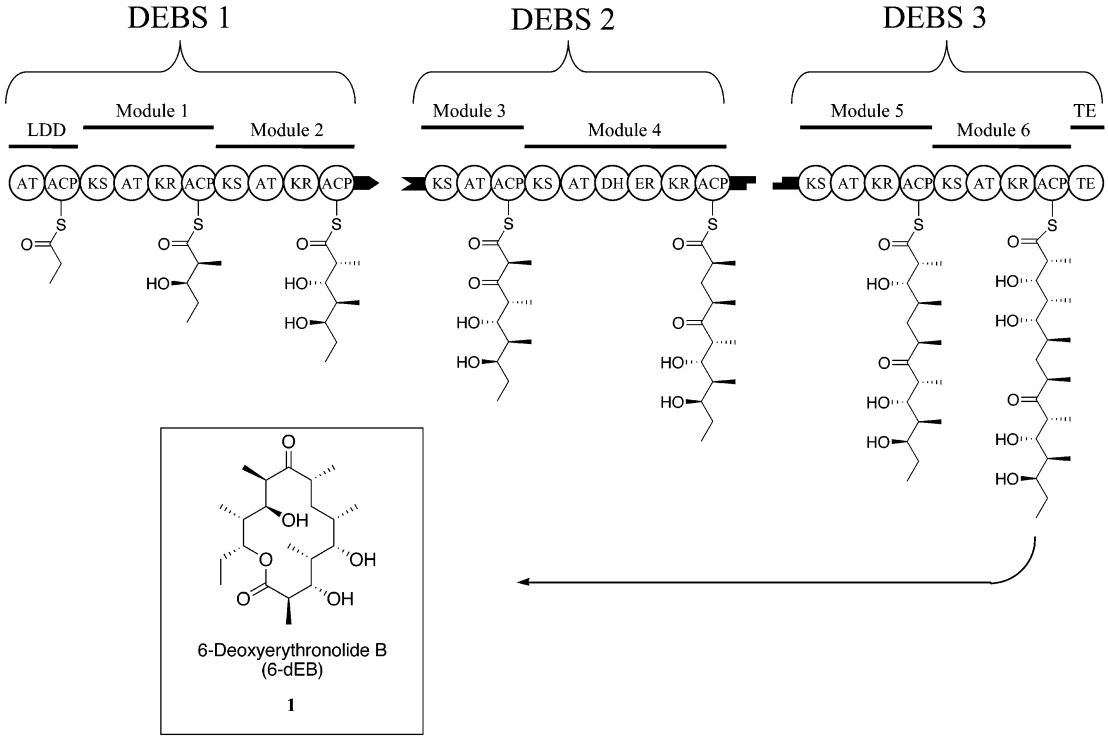

Figure 1. Biosynthesis of 6-deoxyerythronolide B (6dEB) by deoxyerythronolide B synthase (DEBS). DEBS consists of three large polypeptides (DEBS 1−3, each M > 300 kDa). The biosynthetic pathway is primed by a propionyl unit, which is extended in a stepwise manner via decarboxylative condensations with six methylmalonyl-extender units. The thioesterase domain located at the C-terminal end of DEBS 3 catalyzes macrolactone formation leading to 6dEB (compound 1). Each chain elongation cycle plus subsequent reduction steps is catalyzed by a set of domains designated as a module. A module is comprised of a β-keto synthase domain (KS), an acyl transferase domain (AT), an acyl carrier protein (ACP), and optionally a keto reducatse (KR), dehydratase (DH), and/or enoyl reductase (ER) domain.

been exploited for the engineered biosynthesis of numerous 6dEB and erythromycin analogues by domain inactivation, insertion, or substitution.[4] Most notably, individual methylmalonyl-specific AT domains of DEBS have been replaced by malonyl-specific, ethylmalonyl-specific, or methoxymalonyl-specific AT domains from other PKSs to yield the expected regioselectively modified polyketide products.[5−10] Notwithstanding these successes, in most cases, recombinant bacteria that produce such "hybrid" polyketides yield substantially reduced quantities of product relative to their wild-type counterparts. The mechanistic basis for this imperfection has not been explored and could be due to several reasons (or combinations thereof). For example, reduced polyketide productivity could result from attenuated steady-state intracellular concentrations of the hybrid PKS, possibly due to folding defects or proteolytic susceptibility of the engineered protein. Alternatively, it could be due to the inability of the heterologous domain to recognize other domains that it must interact with during the catalytic cycle of the module. It could also result from specificity constraints at other catalytic sites within the hybrid module or in downstream modules. Finally, the introduction of a heterologous domain into a module could perturb the protein−protein interactions that are crucial for enabling efficient interdomain catalysis. To address these possibilities, we undertook detailed biochemical investigations into the properties of a set of hybrid derivatives of module 6 of DEBS, in which the natural AT domain is replaced with homologues from other DEBS modules or other PKS modules (Figure 2).

Results

Expression, Purification, and Preliminary Characterization of Derivatives of DEBS Module 6+TE in Which the AT Domain Has Been Replaced with Other Homologous Domains. The methylmalonyl-specific AT domain of DEBS module 6+TE was replaced with homologous AT domains from modules 4 and 5 of DEBS (both methylmalonyl-specific) as well as from module 2 of RAPS, which is malonyl-specific. The resulting hybrid modules had several noteworthy properties. First, the optimal expression conditions for the hybrid modules were substantially altered as compared to wild-type module 6+TE. Whereas the latter enzyme was highly expressed in *Escherichia coli* at an induction temperature of 25−30 °C (10−15 mg of soluble protein per liter of culture volume), the engineered modules were exclusively produced as insoluble proteins under these conditions. Therefore, an induction temperature of 13 °C was employed for the hybrid modules, which led to production levels of 0.5−1 mg of soluble protein per liter of culture volume. Similarly, purification of the wild-type DEBS module 6+TE was achieved using Ni-NTA resin, resulting in a homogeneous protein with approximately 95%

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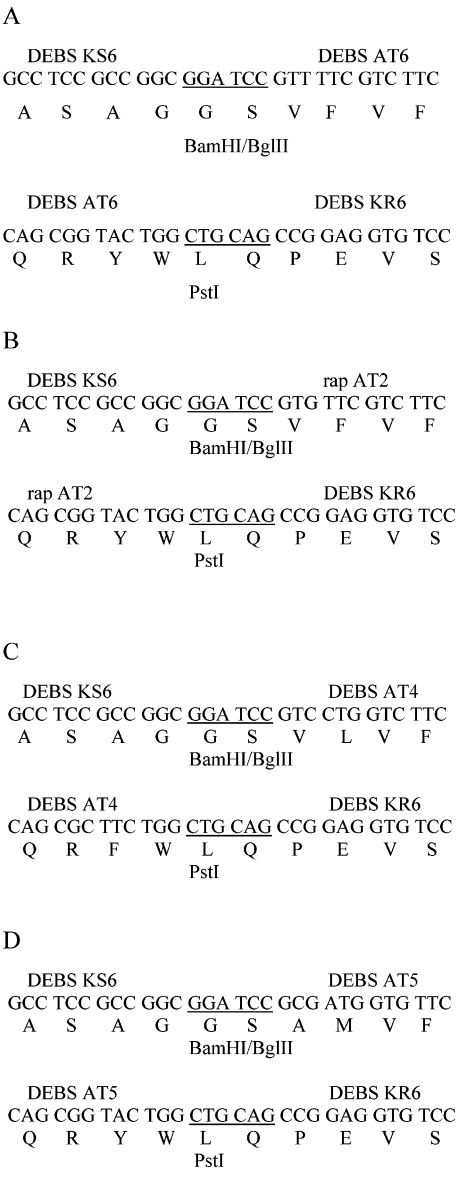

Figure 2. Locations of domain junctions of hybrid DEBS module 6+TE derivatives containing heterologous AT domains. (A) *Bam*HI/*Pst*I restriction sites in pJL459, which expresses module 6+TE. (B) Domain junctions in pJL458, in which the malonyl-specific AT-2 domain of RAPS has been introduced in place of the methylmalonyl-specific AT domain of module 6+TE. (C) Domain junctions in pMH9, in which the methylmalonyl-specific AT domain of module 6+TE has been replaced with another methylmalonyl-specific AT domain from DEBS module 4. (D) Domain junctions in pMH10, in which the methylmalonyl-specific AT domain of module 6+TE has been replaced with another methylmalonyl-specific AT domain from DEBS module 5.

purity. However, all of the hybrid modules exhibited a significantly altered binding affinity toward Ni-NTA resin, suggesting altered protein conformation. While module 6(RAPS AT2)+TE could be purified to >90% homogeneity, module 6(DEBS AT4)+TE and module 6(DEBS AT5)+TE preparations required additional purification steps such as HiTrap Q anion-chromatography and size exclusion chromatography on Superdex 200 resin (Figure 3). Notwithstanding these differences, both wild-type and hybrid proteins had apparent molecular masses of ~400 kDa (data not shown), which corresponds to a dimeric native state. As reported earlier, a dimeric quaternary structure is a crucial prerequisite of module activity.[11]

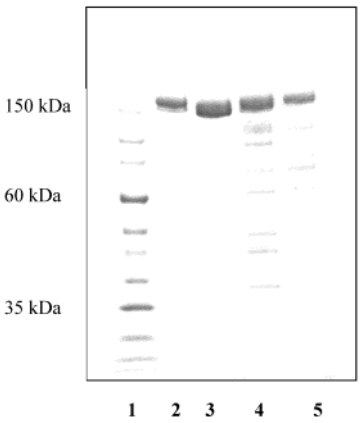

Figure 3. SDS-PAGE (4−15% polyacrylamide) of purified DEBS module 6+TE and engineered derivatives. Lane 1, molecular mass marker; lane 2, wild-type DEBS module 6+TE; lane 3, DEBS module 6 (RAPS AT2)+TE; lane 4, DEBS module 6 (DEBS AT4)+TE; lane 5, DEBS module 6 (DEBS AT5)+TE.

Steady-State Kinetic Analysis of Derivatives of DEBS Module 6+TE in Which the AT Domain Has Been Replaced with Other Homologous Domains. The relative abilities of wild-type DEBS module 6+TE, module 6(RAPS AT2)+TE, module 6(DEBS AT4)+TE, and module 6(DEBS AT5)+TE to catalyze chain elongation were measured by analyzing steady-state rates of formation of triketide lactone products obtained from NDK, the natural diketide substrate of DEBS module 2. (In earlier studies [see ref 12], it has been shown that NDK is an excellent substrate of DEBS module 6+TE, although the high K_M for this substrate precludes saturation under typical experimental conditions.) At an NDK concentration of 5 mM, the maximum velocity (with respect to methylmalonyl-CoA) of DEBS module 6+TE was 0.7 min^{-1}, whereas both module 6(RAPS AT2)+TE and module 6(DEBS AT4)+TE had corresponding maximum velocities in the range of 0.02−0.04 min^{-1}. The turnover number of module 6(DEBS AT5)+TE was comparably attenuated, but could not be reliably quantified due to limitations associated with the maximum attainable concentration of this enzyme in the assay mixture.

To pinpoint the sources of impairment in the hybrid PKS modules, the transacylation activities of the AT domains of DEBS module 6+TE, module 6(RAPS AT2)+TE, module 6(DEBS AT4)+TE, and module 6(DEBS AT5)+TE were measured. All modules were in the holo-form. Our studies on wild-type module 6+TE as well as several AT-ACP didomains revealed no effect on transacylation rates depending on the modification of the ACP domain.[13] For this purpose, a transacylation assay was employed in which the rate of malonyl or methylmalonyl transfer from the corresponding acyl-CoA to *N*-acetylcysteamine (HS-NAc) was measured.[13] In each case, the k_{cat} for the cognate acyl-CoA:HS-NAc acyl transfer was in the range of 20−40 min^{-1}, and the K_M was 0.1−0.2 mM. Thus, AT domain exchange does not appear to impair the intrinsic catalytic activity of the incoming AT domain.

To investigate whether the acyl-CoA:ACP acyl transfer activity of the hybrid modules was altered relative to their wild-

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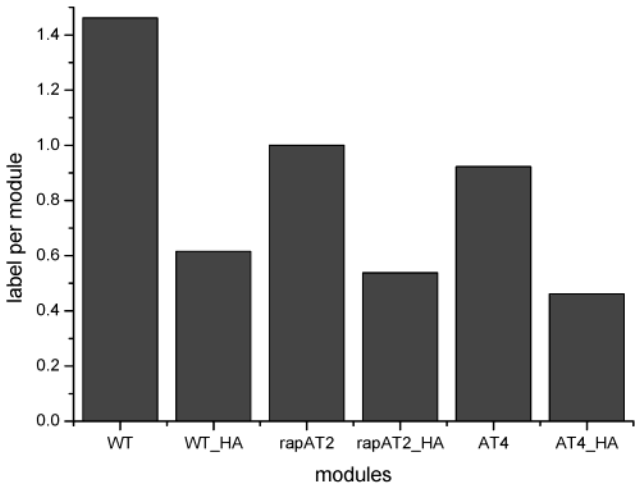

Figure 4. Labeling of DEBS module 6+TE, module 6(RAPS AT2)+TE, and module 6(DEBS AT4)+TE with ^{14}C-methylmalonyl-CoA or ^{14}C-malonyl-CoA. The bars WT and WT_HA correspond to untreated and hydroxylamine-treated wild-type module 6+TE. Similarly, the bars rapAT2, rapAT2_HA, AT4, and AT4_HA correspond to untreated and hydroxylamine-treated hybrid modules.

type counterpart, labeling experiments using ^{14}C methylmalonyl-CoA or ^{14}C malonyl-CoA were conducted as described in the Materials and Methods section. As shown in Figure 4, both the AT and the ACP domains of DEBS module 6+TE, module 6(RAPS AT2)+TE, and module 6(DEBS AT4)+TE are rapidly labeled. In each case, steady-state levels of occupancy are achieved in <30 s. The maximum labeling intensity for the engineered modules is approximately 60−70% that of the intensity for wild-type DEBS module 6+TE. To compare the extent of extender unit transfer to the ACP domains, each labeled protein was first denatured and subsequently incubated with 0.3 M hydroxylamine, which specifically cleaves acyl-S-ACP thioester linkages while leaving acyl-O-AT linkages unaffected. Thus, the decrease in labeling intensity can be correlated to the steady-state occupancy of each ACP domain by the incoming acyl group. As shown in Figure 4, approximately 50% of the ACP is labeled in each case, suggesting that the AT → ACP acyl transfer capability is largely intact in the hybrid PKS modules.

Pre-Steady-State Kinetic Analysis of Wild-type and Hybrid Modules. Because AT → ACP acyl transfer is fast as compared to the overall rate of chain elongation catalyzed by a PKS module,[12,13] the intrinsic impact of charging the ACP of a PKS module with an unnatural extender unit cannot be decoded by steady-state kinetic analysis of modules. If the rate of chain elongation (which, under steady-state conditions, is controlled by the KS or one of the downstream β-ketoacyl chain modifying enzymes) is indeed affected by the choice of the extender unit, then this would represent a fundamental barrier in the biosynthesis of unnatural polyketides, which could not be overcome by engineering AT domains. For example, in the related nonribosomal peptide synthetases, the condensing enzyme is known to have specificity toward its natural extender unit.[14] In addition to probing the intrinsic nucleophilic substrate specificity of KSs, pre-steady-state kinetic analysis could also be used to investigate the impact of AT domain substitution on chain elongation independent of AT → ACP acyl transfer. However, probing nucleophile specificity of PKS modules via pre-steady-state kinetic analysis is challenging, because acyl-ACP intermediates are rapidly hydrolyzed in the absence of KS-bound ketide chains.[13,15,16]

To overcome this problem, the AT domains of DEBS module 6+TE, DEBS module 2+TE, and DEBS module 6(RAPS AT2)+TE were inactivated by Ser-Ala mutations at their AT active sites. As expected, the ACP domains of the resulting PKS modules could be readily charged by direct phosphopantetheinylation of the apo-protein in the presence of a suitable acyl-CoA substrate. More significantly, the acyl-ACPs of these precharged PKS proteins did not undergo rapid hydrolysis. Because a Ser → Ala mutation is likely to introduce minimal perturbations into the overall PKS structure, precharged PKS proteins could therefore be used to quantify nucleophile specificity (if any) associated with the chain elongation portion of the overall catalytic cycle.

Figure 5A shows the products of a typical single turnover burst-experiment analyzed via radio-TLC. At 5 mM NDK, the calculated rate constant for chain elongation by DEBS module 6+TE was 0.7 min^{-1}. This number compares well with the steady-state turnover number of the same wild-type module under similar conditions (see above) and suggests that acylation of the KS by the electrophile was not rate-limiting in this pre-steady-state kinetic assay. Remarkably, both DEBS module 2+TE and DEBS module 6+TE showed comparable rates of triketide lactone formation (0.7−0.8 min^{-1}) if either methylmalonyl-CoA or malonyl-CoA were precharged on the ACP (Figure 5B). Thus, the high specificity of these modules for methylmalonyl extender units appears to be controlled exclusively by their AT domains. Notably, because the maximum number of achievable turnovers with these modules approaches the stoichiometric limit, it also appears that racemic (2*RS*)-methylmalonyl groups bound to the active site of the ACP are resolved, either prior to the condensation reaction or immediately following β-ketoacyl-ACP formation. In contrast, the rates of triketide lactone formation by DEBS module 6(RAPS AT2)+TE were below detectable limits in the presence of either malonyl- or methylmalonyl-CoA. The kinetics of acylating the KS domains of the three modules with radiolabeled NDK were compared and were found to be similar (Figure 6), suggesting that the problem with chain elongation in DEBS module 6(RAPS AT2)+TE is not associated with chain transfer of the electrophile to the KS. Together, these findings suggest that impaired chain elongation properties of AT-exchanged modules are due to altered interactions between the ACP and KS domains.

AT Domain Exchange Causes Significant Conformational Changes in DEBS Module 6(RAPS AT2)+TE. To obtain protein chemical evidence for possible effects of AT domain exchange on the module conformation, limited proteolysis was conducted on DEBS module 6+TE and module 6(RAPS AT2)+TE using trypsin. As can be seen in Figure 7, the latter module is more sensitive toward trypsin digest. In particular, two abundant fragments unique to this hybrid module were of interest (~120 kDa and ~40 kDa). N-terminal sequencing of

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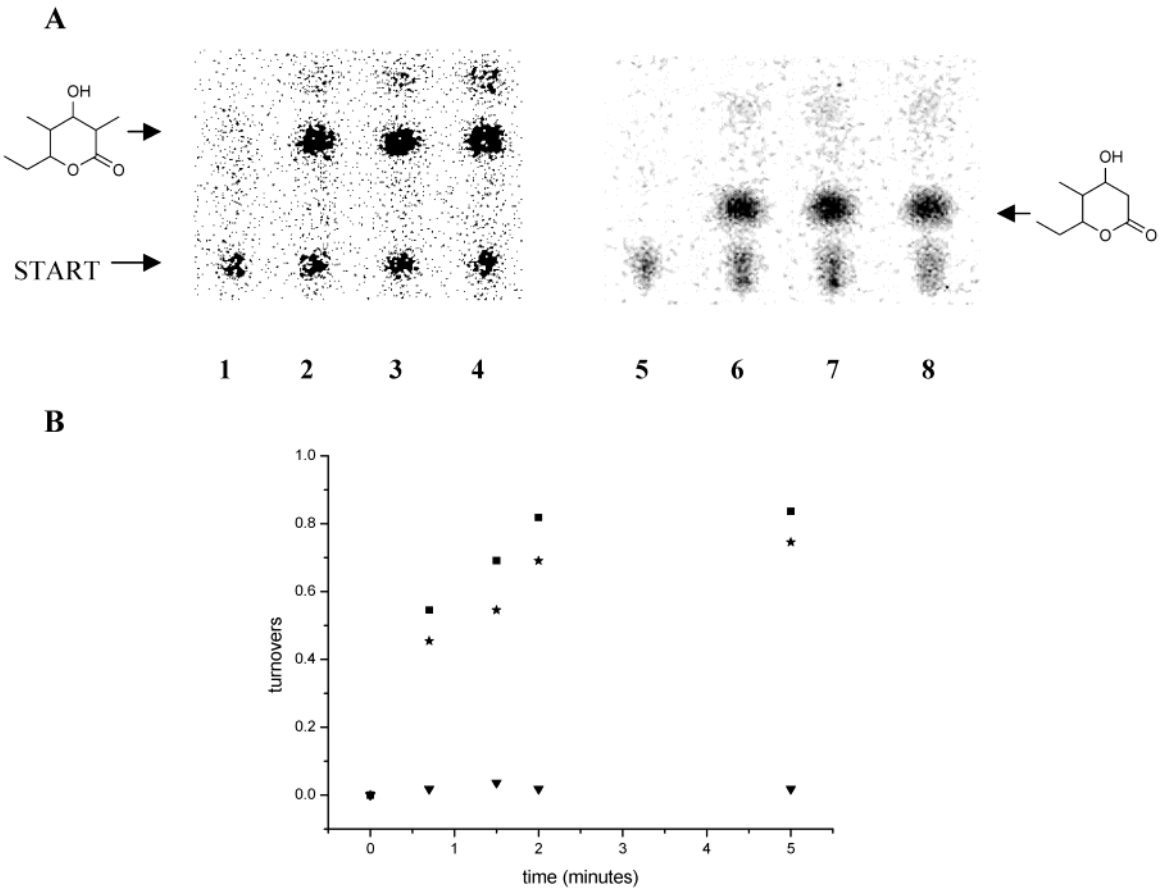

Figure 5. Single turnover kinetics of DEBS module 6+TE and DEBS module 6(rapAT2)+TE. For performing single turnover experiments, the apo-modules, which harbored Ser-Ala inactivated AT domains, were preloaded with extender-CoAs using the *sfp* protein as described in Materials and Methods. Single turnover was carried out in 400 mM phosphate buffer pH 7.2, 20% glycerol, 1 mM DTT, 1 mM EDTA. After addition of 10 μM preloaded module and 10 mM NADPH, the reaction was started with 5 mM NDK-SNAc. (A) Autoradiograms of typical single turnover experiments. Visualization of the triketide lactone products by thin-layer chromatography. Lanes 1−4: Time points of single turnover experiment with preloaded methylmalonyl-extender unit at 0 (no NDK-SNAc added), 1, 2, and 4 min. Lanes 5−8: Time point of single turnover experiment with preloaded malonyl-extender unit at 0 (no NDK-SNAc added), 1, 2, and 4 min. (B) Graph of single turnover bursts with DEBS module 6+TE (AT Ser-Ala) (■ and ★) and DEBS module 6(rap AT2 Ser-Ala)+TE (▼). ■: Single turnover burst with preloaded methylmalonyl-extender unit. ★: Single turnover burst using preloaded malonyl-extender unit. ▼: Single turnover burst with preloaded methylmalonyl-extender units. The data clearly show that there is comparable turnover of DEBS module 6 (AT Ser-Ala)+TE when preloaded with methylmalonyl- or malonyl-extender unit. In contrast, when module 6 (rapAT2 Ser-Ala)+TE was preloaded with methylmalonyl-extender units, no product formation could be detected. This suggests that no condensation reaction took place.

the 40 kDa fragment was not possible, perhaps due to a blocked N-terminal residue. However, LC-MS analysis of this fragment revealed a molecular mass of 41 280 Da, which agrees well with the SDS-PAGE results. Furthermore, MALDI-MS analysis identified it as being composed of ACP and TE domain (including the C-terminal hexahistidine tag of the protein) (Figure 8). Thus, introduction of a heterologous AT domain renders the module more sensitive to trypsin at a site that is located between the KR and the ACP domains (i.e., C-terminal to the actual site of introduction of the AT domain by several hundred amino acids). This finding is suggestive of a conformational change in the hybrid module.

Discussion

Engineering of modular polyketide synthases (PKSs) is emerging as a powerful approach to generate unnatural natural products with altered biological activities, including antiinfective, anticancer, and other pharmacological agents.[17−20] Such engineering often relies on deleting, replacing, or adding individual catalytic domains in one or more modules within a PKS scaffold. However, these structurally and functionally altered modules often lead to attenuated productivity of the engineered polyketide product. Impaired activity most likely stems from structural perturbations and/or changes in catalytic specificity, although the causes for impairment have not been elucidated in any system thus far. The goal of this work was to understand the precise implications of AT domain exchange, as currently practiced, on PKS module structure and activity.

AT domains are widely recognized as the primary gatekeepers for building blocks for polyketide biosynthesis. For example, numerous studies have shown that the structure of a polyketide can be regioselectively altered by AT domain substitution.[5−8,10,16,21−24] However, in nearly all cases, manipulation of the AT domain causes a major reduction in polyketide produc-

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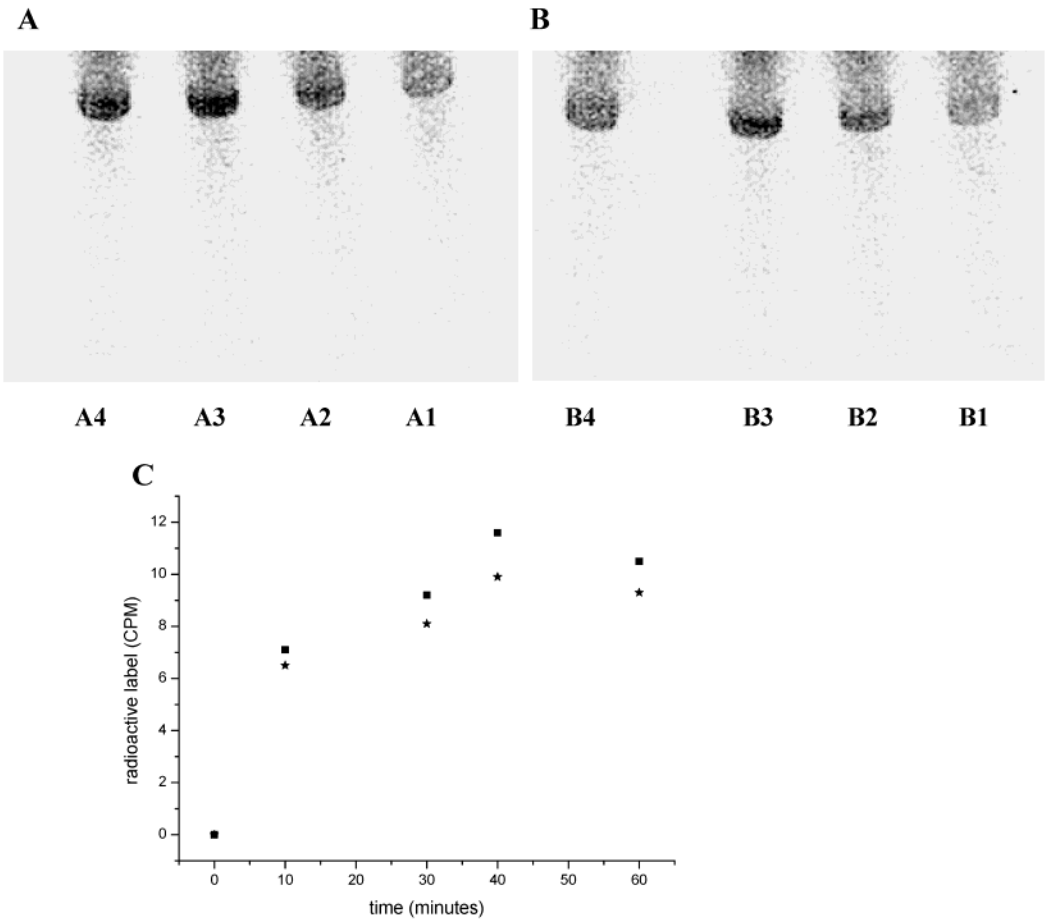

Figure 6. Labeling kinetics of wild-type DEBS module 6+TE and DEBS module 6(RAPS AT2)+TE with ^{14}C-labeled diketide. A and B: Autoradiograms of SDS-PAGE gel. Each lane contains 50 pmol of module protein. Lanes A1−4: Time course of labeling wild-type DEBS module 6+TE. Lanes B1−4: Time course of DEBS module 6 (RAPS AT2)+TE. (C) The time courses from A and B are plotted. The data show comparable labeling kinetics of both modules, suggesting that the β-ketosynthase domains in wild-type DEBS module 6+TE as well as in DEBS module 6 (rapAT2)+TE are comparably capable of loading the growing polyketide chain.

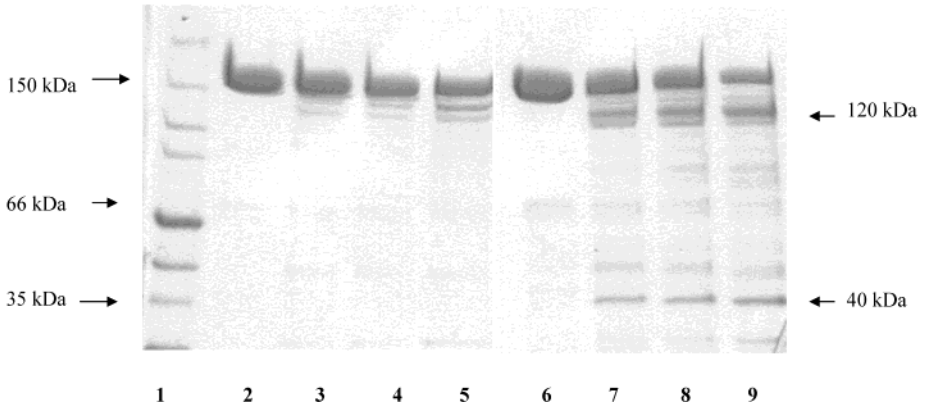

Figure 7. SDS-PAGE analysis of limited proteolysis of wild-type DEBS module 6+TE and DEBS module 6(RAPS AT2)+TE. Lane 1: Broad range molecular mass marker proteins. Lanes 2−5: Wild-type DEBS module 6+TE (5 μg) incubated with 50 ng of porcine pancreas trypsin; time points taken at 0, 1, 2, and 5 min. Lanes 6−9: DEBS module 6 (RAPS AT2)+TE (5 μg) incubated with 50 ng of porcine pancreas trypsin; time points taken at 0, 1, 2, and 5 min. A ~40 kDa fragment is generated more rapidly from the hybrid module as compared to the wild-type module.

tivity. To understand the nature of this problem, we constructed, expressed, and purified a set of wild-type and hybrid PKS modules and established a panel of assays to quantitatively probe their biochemical properties.

A priori, such problems could be due to either in vivo proteolytic susceptibility and/or intrinsic kinetic defects in the multidomain catalyst. By analyzing individually purified wild-type and hybrid modules, we have systematically investigated the potential sources of kinetic problems. One might expect kinetic bottlenecks in these hybrid modules to result from (i) reduced acyl transferase activity of heterologous AT domains; (ii) inability of heterologous AT domains to efficiently recognize the acceptor ACP in the host module; (iii) acyl group-dependent proofreading mechanism(s) in the acyl-CoA:ACP acyl transfer

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Identified Fragment	Observed mass (g/mol)	Computed mass (g/mol)
LAAALEHHHHHH	1408.77	1408.68
LAGALR	599.36	599.37
GIAPVR	611.37	611.37
LTALGAYDR	978.57	978.51
LTGQWRPR	1012.62	1012.57
HIDAWLGGGNSSSVDK	1641.96	1641.77
AVPQPGYEEGEPLPSSM-AAVAAVQADAVIR	3040.34	3040.40
NQLQQATGLALPATLV-FEHPTVR	2505.10	2504.36

Figure 8. Sequence analysis of the 40 kDa fragment obtained via limited proteolysis of DEBS module 6 (RAPS AT2)+TE. Identified peptide fragments derived from the 40 kDa fragment. The protein was eluted from a SDS-PAGE gel and completely digested with trypsin. The resulting peptides were analyzed by LC-MS and mapped onto the hybrid module via standard sequence analysis.

reaction; or (iv) barriers to the recognition of unnatural extender units during chain elongation or β-ketoacyl chain modification. Remarkably, however, on the basis of our analysis of a set of representative hybrid modules, none of these potential problems appear to present a serious bottleneck. Rather, the introduction of the heterologous AT domain appears to damage preexisting interactions between the KS and ACP domains, which must associate during the catalysis of C−C bond formation. (While an effect of the heterologous AT domain on the KR-catalyzed reaction cannot be ruled out, this is unlikely because the rates of both β-hydroxy triketide lactone and β-keto-lactone formation are reduced.) Presumably, this destabilization also negatively impacts expression levels of soluble hybrid proteins. Thus, domain swaps appear to induce changes in regions of the module that are outside the exchanged domain itself. Next-generation strategies for altering the specificity of PKS modules will have to solve or circumvent this problem, if improved multimodular PKS catalysts are desired.

Our results described here also reveal another fundamental property of PKS modules that has considerable significance for combinatorial biosynthesis. Whereas the importance of AT domains in determining extender unit selectivity is well documented, the existence of other checkpoints within a module for unnatural extender units was unknown. Pre-steady-state experiments on both DEBS module 2+TE and module 6+TE have demonstrated that no such checkpoints exist in either module for discriminating between methylmalonyl extenders (the natural substrate of these modules) and malonyl extenders. Thus, if efficient protein engineering strategies can be devised for charging modules with unnatural extender units, it may be possible to generate functionally altered PKSs (and, by inference, structurally altered polyketides) with catalytic properties that approach their natural counterparts.

Materials and Methods

Materials. ^{14}C-labeled substrates (malonyl-CoA and methylmalonyl-CoA) were from ARC radiochemicals. All other chemicals were from Sigma or Fluka (St. Louis, MO). SDS-PAGE gradient gels (4−15% acrylamide gels) were from Bio-Rad (Hercules, CA). The protein molecular mass standard (broad range marker) was from New England BioLabs. The plasmids pET21c and pET28c were from Novagen. Ni-NTA affinity resin was from Qiagen Inc. TLC plates were obtained from J. T. Baker (Phillipsburg, NJ). HiTrapQ-columns and Superdex-200 size exclusion resin as well as the FPLC systems were from Pharmacia Biotech (Piscataway, NJ).

Bacteria. *E. coli* XL1-blue and *E. coli* BL21 (DE3) were from Stratagene (Cedar Creek, TX) and were manipulated and grown using standard biochemical and molecular biological techniques as described elsewhere. For recombinant synthesis of holo-PKS proteins in which the acyl carrier protein is posttranslationally modified with a phosphopantetheine molecule, *E. coli* BL21 (DE3) BAP1 strain, which contains a chromosomal copy of the *Bacillus subtilis* phosphopantetheinyl transferase gene *sfp*, was used.[25,26] For production of apo-polyketide synthase proteins, *E. coli* BL21 (DE3) was used.

Plasmids. Plasmids for the production of DEBS module 6+TE derivatives in which AT domains had been exchanged were constructed using the following strategy. As described earlier,[9] *Bam*HI/*Pst*I restriction sites were introduced in the flanking sequence regions of AT-6 in pRSG54,[27] which expresses DEBS module 6+TE. The AT domain in the resulting plasmid, pJL459, was then replaced with a *Bgl*II/*Pst*I fragment encoding the homologous AT-2 from the rapamycin synthase (RAPS), giving rise to pJL458. Alternatively, homologous AT-4 and AT-5 domains from DEBS were inserted as *Bam*HI/*Pst*I cassettes, giving rise to pMH9 and pMH10, respectively. For precise locations of the engineered domain boundaries, see Figure 2. Plasmid pRSG64[27] was used to produce DEBS module 2+TE. pMH28 and pMH18 are derivatives of pRSG64 and pRSG54, respectively, in which the active site serine residues (S2598 and S2107, respectively; numbering based on the sequences of the full DEBS proteins[3,28]) were mutated into alanine residues. Similarly, the active site serine residue in module 6(RAPS AT2)+TE encoded by pJL458 (S3872 in RapA[29]) was mutated into an alanine residue, yielding pMH19. Mutagenesis was performed using the Quikchange kit (Stratagene). The following oligonucleotides were added in the reactions. For pRSG54: primer 1a, CGGCCGTCATCGGCCAT**GCG**CAGGGCGAGATC; primer 1b, GATCTCGCCCTGCGCATGGCCGATGACGGCCG. For pJL458: primer 2a, GATGCGGTGGTCGGTCAC**GCT**GTCGGTGAGCTC; primer 2b, GAGCTCACCGACAGCGTGACCGACCACCGCATC. For pRSG64: primer 3a, GCCGTCATAGGGCAC**GCG**CAGGGTGAGATCGCC; primer 3b, GGCGATCTCACCCTGCGCGTGCCCTATGACGGC. The following changes were made to the protocol described by the vendor: 150 ng of template plasmid was added to the mixture. The PCR reaction was carried out using 3 min extension time per kb plasmid length. Additionally, the *Dpn*I treatment was performed for 3 h with 2 μL of enzyme supplied with the kit.

Expression and Purification of DEBS Module 6+TE, DEBS Module 6+TE (S2107A), and DEBS Module 2+TE (S2598A). *E. coli* cells BAP1 harboring pRSG54, pMH18, or pMH28 were grown in 1 L of LB broth at 37 °C until a OD_{600} of 0.5−0.7 was reached. The culture was cooled at 4 °C for 30 min, and protein synthesis was subsequently induced by addition of 50 μM IPTG. After the flask was shaken at 250 rpm for 12 h at 25 °C, cells were harvested and resuspended in 50 mL of lysis buffer (100 mM phosphate pH 8.0, 20% glycerol, 0.2 M NaCl, 10 mM imidazole, 1 mg/L leupeptin, 1 mg/L pepstatin A, 1 mM benzamidine). The bacteria were lysed using sonication (Branson sonifier, Branson Ultrasonics, Danbury, CT) at a power of 80 W under cooling with ice. Cell debris was removed by centrifugation at 20 000*g*, 4 °C, 60 min. Subsequently, DNA was precipitated by addition of 0.1% PEI. After an additional centrifugation step, 4 mL of a 50% Ni-NTA slurry was added to the supernatant, and the solution was stirred at 4 °C for 1 h. The mixture was loaded into a column using gravity flow. After the resin was washed with 20 mL

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of wash buffer (100 mM phosphate pH 8, 20% glycerol, 0.2 M NaCl, 20 mM imidazole), the protein was eluted with 5 column vol of elution buffer (wash buffer + 200 mM imidazole and 2 mM DTT). Protein-containing fractions were collected and concentrated using Centriprep filter devices (Amicon Inc.) giving a final concentration of 2−10 mg/mL. The enzyme was frozen in liquid nitrogen and stored at −80 °C.

Expression and Purification of DEBS Module 6 (RAPS AT2)+TE and DEBS Module 6 (RAPS AT2 Ser-Ala)+TE. *E. coli* BAP1 harboring pJL458 or pMH19 were grown in LB broth at 37 °C until a OD_{600} of 0.7−1.0 was reached. The culture was cooled at 4 °C for 30 min. After addition of 50 μM IPTG, the cells were incubated in a shaker at 13 °C for 40 h. After harvesting, cells were resuspended in lysis buffer (100 mM phosphate pH 8.0, 20% glycerol, 0.2 M NaCl, 5 mM imidazole, 1 mg/L leupeptin, 1 mg/L pepstatin A, 1 mM benzamidine) and lysed using sonication at a power of 80 W under cooling with ice. Cell debris was removed by centrifugation at 20 000*g*, 4 °C, 60 min, and Ni-NTA affinity chromatography purification was performed for the module 6+TE as described above.

Expression and Purification of DEBS Module 6 (DEBS AT4)+TE. The gene encoding module 6(DEBS AT4)+TE was expressed as described for module 6(RAPS AT2)+TE. Cells were harvested and lysed in lysis buffer (100 mM phosphate pH 8.0, 20% glycerol, 0.2 M NaCl, 1 mg/L leupeptin, 1 mg/L pepstatin A, 1 mM benzamidine) by sonication. After a 0.1% PEI precipitation step, the clear supernatant was combined with 4 mL of a 50% Ni-NTA slurry and stirred at 4 °C for 2 h. The mixture was loaded under gravity flow onto a column. The resin was washed with 100 mM phosphate pH 8.0, 0.2 M NaCl. Elution of the proteins was performed with wash buffer containing 200 mM imidazole and 2 mM DTT. Fractions harboring the module 6 (DEBS AT4)+TE were pooled and applied onto a HiTrapQ-column (5 mL, Pharmacia Biotech), which was preequilibrated with 100 mM phosphate pH 8.0, 2 mM DTT, 20% glycerol. After the column was washed with 5 vol of equilibration buffer, proteins were eluted with a linear gradient of 0−0.5 M NaCl in equilibration buffer. Module 6(DEBS AT4)TE thus obtained was sufficiently pure (>90% homogeneity) for further biochemical experiments. The enzyme was concentrated using a centriprep filter (Amicon) to a final concentration of 10−15 mg/mL and was stored at −80 °C for further use.

Expression and Purification of DEBS Module 6 (DEBS AT5)+TE. Module 6 (DEBS AT5)+TE was expressed and purified according to the procedure described for module 6 (DEBS AT4)+TE. However, after elution from a HiTrapQ column and concentration to a volume of 0.2−0.3 mL, further purification was obtained on a Superdex 200 size exclusion chromatography column. This column was preequilibrated with 100 mM phosphate pH 8, 150 mM NaCl, 2 mM DTT.

Enzyme Assays. The assay procedure for triketide lactone formation by DEBS module 6+TE has been described earlier.[12] The natural diketide substrate of module 2 of DEBS ((2*S*,3*R*)-2-methyl-3-hydroxypentanoyl-SNAc, NDK) is also an excellent substrate for DEBS module 6+TE.[12] It was incubated at a concentration of 5 mM with 5 mM NADPH, 1−5 μM PKS, and 0.5 mM ^{14}C-labeled extender unit (methylmalonyl-CoA or malonyl-CoA) in 400 mM phosphate buffer pH 7.2, 20% glycerol, 2 mM DTT, 1 mM EDTA at 30 °C. Individual time points were taken by quenching 20 μL of the reaction mixture in 200 μL of ethyl acetate, followed by extraction of the triketide lactone product. The product was quantified by radioactive thin-layer chromatography (radio-TLC) in ethyl acetate:hexanes 1:1 followed by autoradiography (Packard Instant Imager).

Determination of the Apparent Native Molecular Masses of the Module Proteins. To determine the apparent molecular masses of the PKS proteins, size exclusion chromatography was performed. An analytical Superdex 200 high performace column was equilibrated with 100 mM phosphate buffer pH 8.0, 150 mM NaCl, 2 mM DTT. The column was calibrated using the High Molecular Weight Calibration Kit from Pharamicia Amersham. Samples were applied in a final volume of 100 μL.

Labeling of Apo- and Holo-Modules. PKS proteins (10 μM, in 100 mM phosphate buffer pH 7.2, 20% glycerol, 1 mM EDTA) were incubated on ice with 100 μM ^{14}C-labeled methylmalonyl-CoA or malonyl-CoA. Time points were taken by quenching the reaction mixture with 1 vol of SDS-PAGE loading buffer lacking any thiol reagents such as DTT or mercaptoethanol. Samples were heated at 70 °C for 5 min and loaded onto a SDS-PAGE gel. The gel was dried using a BioRad gel drying system and analyzed using a phosphoimager. To distinguish betweeen labeling of the AT and ACP domains, samples were denatured as described above, but subsequently mixed with 0.3 M hydroxylamine at pH 8.5 and incubated for 30 min at 30 °C. Hydroxylamine specifically cleaves thioesters but does not affect oxoester bonds. Thus, labeled probes attached to the pantetheinyl arm of the ACP domain are cleaved, whereas those attached to the active site serine residue of the AT domain are not removed.

Measurement of Transacylation Activity of AT Domains. AT domains from modular PKSs can transform their cognate acyl substrates from CoA thioesters to the corresponding *N*-acetyl-cysteamine thioester.[13] To quantify this property, 0.5−1 μM PKS holo-module protein was incubated with 10 mM *N*-acetyl-cysteamine (HS-NAc) and 50−500 μM ^{14}C-labeled methylmalonyl-CoA or malonyl-CoA. The sample was buffered in 100 mM phosphate pH 7.0, 20% glycerol, and the reaction temperature was 20 °C. Individual time points were collected by quenching 10 μL sample volumes in 25 μL of acetone. The mixtures were directly applied to thin-layer chromatography using the conditions of 89% ethyl acetate, 10% 2-propanol, and 1% trichoroacetic acid, and acyl-S-NAc products were quantified on a phosphoimager.

Single Turnover Kinetic Analysis of DEBS Module 6+TE, DEBS Module 2+TE, and DEBS Module 6(RAPS AT2)+TE. Because the presence of a catalytically active AT domain in a module results in rapid hydrolysis of a malonyl- or methylmalonyl-ACP intermediate, single turnover kinetics of modules were measured using mutant forms of DEBS module 6+TE, DEBS module 2+TE, and DEBS module 6(RAPS AT2)+TE in which the active site serine residues in the corresponding AT domains had been altered into alanine residues, as described above. For single turnover assays, the modules were preloaded with ^{14}C-labeled malonyl- or methylmalonyl-extender units by incubating the module (30 μM) with 10 mM $MgCl_2$, 50 μM methylmalonyl-CoA or malonyl-CoA (60 mCi/mmol specific activity for both compounds), and 0.1 μM Sfp^{26} in 100 mM phosphate buffer, pH 6.6, at 30 °C for 30 min. Stoichiometric labeling of the ACP was confirmed via radioactive SDS-PAGE. Subsequently, the mixture (50−70 μL vol) was adjusted to 400 mM phosphate buffer, pH 7.0, 20% glycerol, 2 mM DTT, 1 mM EDTA, and the chain elongation reaction was started by adding 10 mM NADPH and 5 mM NDK substrate simultaneously. The sample temperature was kept constant at 20−23 °C. Time points were taken every 20 s by quenching 15−25 μL of the sample in 200 μL of ethyl acetate, and the amount of triketide lactone synthesized was measured by radio-TLC as above.

Limited Proteolysis of DEBS Module 6+TE and Module 6(RAPS AT2)+TE. The modules were dissolved in 100 mM phosphate buffer pH 8, 150 mM $MgCl_2$ at a final concentration of 0.1 mg/mL. At 0 °C, 1:100 (w/w) trypsin was added. Time points were taken at 0, 1, 2, 3, and 30 min by quenching 5 μL reaction mixture in 5 μL of SDS-PAGE sample buffer, followed by heating at 70 °C for 5 min. The proteins were separated by SDS-PAGE, and the polypeptides of interest were directly eluted from the gel, subjected to complete trypsin digestion, and analyzed via LC-MS to determine their identity. Direct LC-MS analysis was also performed on a sample of partially proteolyzed module 6(RAPS AT2)+TE after 1 min using a RP-C18 column. The sample volume for injection was 130 μL, and the protein concentration was 1 mg/mL. A linear gradient of 25−70% acetonitrile in water was applied for 34 min. The fragment of interest (40 kDa, see below) eluted at a retention time of 17 min.

In Gel Tryptic Digest for MALDI-Mass Spectrometry. The polypeptide fragment of interest (40 kDa) was extracted from an SDS-

PAGE gel as follows. After a gel slice containing this polypeptide was dried, 10 μL of 45 mM dithiothreitol (DTT) followed by 100 μL of 100 mM Tris/HCl pH 7.8 was added. The mixture was incubated at 55 °C for 30 min. The solution phase was discarded, and 10 μL of a 100 mM acrylamide or iodoacetamide solution was added, followed by 100 μL of 100 mM Tris/HCl pH 7.8. Subsequently, the sample was incubated in the dark for 1 h. The solution phase was again discarded, and the gel was washed with 500 μL of 50 mM Tris/HCl pH 7.8/50% acetonitrile for 30 min. After removal of the buffer, the gel was dried in a Speed Vac apparatus to completion. For complete proteolytic digest, 40 μL of 25 mM Tris/HCl pH 7.8 containing 1 pmol Trypsin was added, and the sample was incubated at 37 °C overnight. For MALDI-mass analysis, a 10 μL aliquot was withdrawn from the reaction solution, and the peptides were extracted using ZipTip columns (μC18 variety). The column was washed with 0.1% TFA, and the resulting peptide fractions were directly eluted onto a MALDI plate using 1 μL of 50% acetonitrile. The eluate was dried partially and supplemented with 0.5 μL of α-cyano-4-hydroxycinnamic acid at a concentration of 10 mg/mL in 0.1% TFA/33% acetonitrile/33% ethanol. MALDI-MS was carried out using deflector mode to obtain monoisotopic masses. The mass map obtained was used to match the protein to theoretical digests of proteins cited in databases such as NCBI nonredundant database. Additionally, usage of the known amino acid sequences of DEBS module 6 and the AT-exchanged derivatives led to unambiguous identification of the tryptic peptides.

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