Biochemical Analysis of the Substrate Specificity of the β-Ketoacyl-Acyl Carrier Protein Synthase Domain of Module 2 of the Erythromycin Polyketide Synthase†

Jiaquan Wu,‡ Kenji Kinoshita,‡ Chaitan Khosla,§ and David E. Cane*,‡

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

Received August 26, 2004; Revised Manuscript Received October 1, 2004

ABSTRACT: The β-ketoacyl-acyl carrier protein synthase (KS) domain of the modular 6-deoxyerythronolide B synthase (DEBS) catalyzes the fundamental chain building reaction of polyketide biosynthesis. The KS-catalyzed reaction involves two discrete steps consisting of formation of an acyl−enzyme intermediate generated from the incoming acylthioester substrate and an active site cysteine residue, and the conversion of this intermediate to the β-ketoacyl-acyl carrier protein product by a decarboxylative condensation with a paired methylmalonyl-SACP. We have determined the rate constants for the individual biochemical steps by a combination of protein acylation and transthioesterification experiments. The first-order rate constant (k_2) for formation of the acyl−enzyme intermediate from [1-^{14}C]-(2*S*,3*R*)-2-methyl-3-hydroxypentanoyl-SNAC (**2**) and recombinant DEBS module 2 is 5.8 ± 2.6 min^{-1}, with a dissociation constant (K_S) of 3.5 ± 2.8 mM. The acyl−enzyme adduct was formed at a near-stoichiometric ratio of ∼0.8:1. Transthioesterification between unlabeled diketide-SNAC **2** and *N*-[1-^{14}C-*acetyl*]cysteamine gave a k_{exch} of 0.15 ± 0.06 min^{-1}, with a K_m for HSNAC of 5.7 ± 4.9 mM and a K_m for **2** of 5.3 ± 0.9 mM. Under the conditions that were used, k_{exch} was equal to k_{-2}, the first-order rate constant for reversal of the acyl−enzyme-forming reaction. Since the rate of the decarboxylative condensation is much greater that the rate of reversion to the starting material ($k_3 \gg k_{-2}$), formation of the acyl−enzyme adduct is effectively irreversible, thereby establishing that the observed value of the specificity constant (k_{cat}/K_m) is solely a reflection of the intrinsic substrate specificity of the KS-catalyzed acyl−enzyme-forming reaction. These findings were also extended to a panel of diketide- and triketide-SNAC analogues, revealing that some substrate analogues that are not converted to product by DEBS module 2 form dead-end acyl−enzyme intermediates.

Deoxyerythronolide B synthase (DEBS)[1] catalyzes the multistep biosynthesis of 6-deoxyerythronolide B (**1**, 6-dEB), the parent macrolide aglycone of the widely used, broad spectrum antibiotic erythromycin A and its semisynthetic derivatives, including clarithromycin and zithromax (Figure 1) (*1*−*4*). This large, multifunctional polyketide synthase (PKS) consists of six protein modules, each of which is responsible for a discrete round of polyketide chain elongation and functional group modification. Each constituent DEBS module is itself composed of a small group of discrete, independently folded domains, each of which carries out a discrete biochemical reaction. All six modules carry an acyl carrier protein (ACP) domain and a dedicated β-ketoacyl-SACP synthase (KS) domain, the latter responsible for the characteristic chain elongation reaction, a decarboxylative acylation involving methylmalonyl-SACP and the acyl group of the growing polyketide chain. The ACP domain is itself primed by an acyl transferase (AT) domain that is specific for methylmalonyl-CoA. Individual modules can also carry stereospecific ketoreductase (KR) domains, while module 4 also harbors additional dehydratase (DH) and enoylreductase (ER) domains. A thioesterase (TE), located at the C-terminus of module 6, catalyzes the lactonization of the mature heptaketide and release of macrolide product 6-dEB. DEBS itself serves as the prototype for the important family of modular polyketide synthases (PKSs) that are responsible for the formation of a wide variety of antibiotic, antitumor, and immunosuppressive metabolites (*5*, *6*).

We have reported extensive *in vitro* investigations of the substrate specificity of a variety of PKS modules (*4*). The most intensively investigated of these multifunctional proteins has been DEBS module 2 carrying an appended thioesterase (TE) domain (module 2+TE) (*7*). This hybrid module can be efficiently expressed in *Escherichia coli* and readily purified to homogeneity in either an apo or holo form, depending upon the coexpression of a suitable phosphopantetheinyl transferase. Incubation of module 2+TE with (2*S*,3*R*)-2-methyl-3-hydroxypentanoyl-SNAC (**2**), the *N*-

† This research was supported by grants from the National Institutes of Health to D.E.C. (GM22172) and C.K. (CA66736).

* To whom correspondence should be addressed. Telephone: (401) 863-3588. Fax: (401) 863-9368. E-mail: David_Cane@brown.edu.

‡ Brown University.

§ Stanford University.

[1] Abbreviations: ACP, acyl carrier protein; AT, acyl transferase; BSA, bovine serum albumin; DEBS, 6-deoxyerythronolide B synthase; DH, dehydratase; ER, enoylreductase; IPTG, isopropyl thio-D-galactopyranoside; KR, ketoreductase; KS, β-ketoacyl-acyl carrier protein synthase; LB, Luria-Bertani; LSC, liquid scintillation counting; Ni−NTA, nickel−nitriloacetic acid; NRPS, nonribosomal peptide synthetase; PKS, polyketide synthase; TCEP, tris(2-carboxyethyl)-phosphine.

10.1021/bi048147g CCC: $27.50
Published on Web 11/25/2004

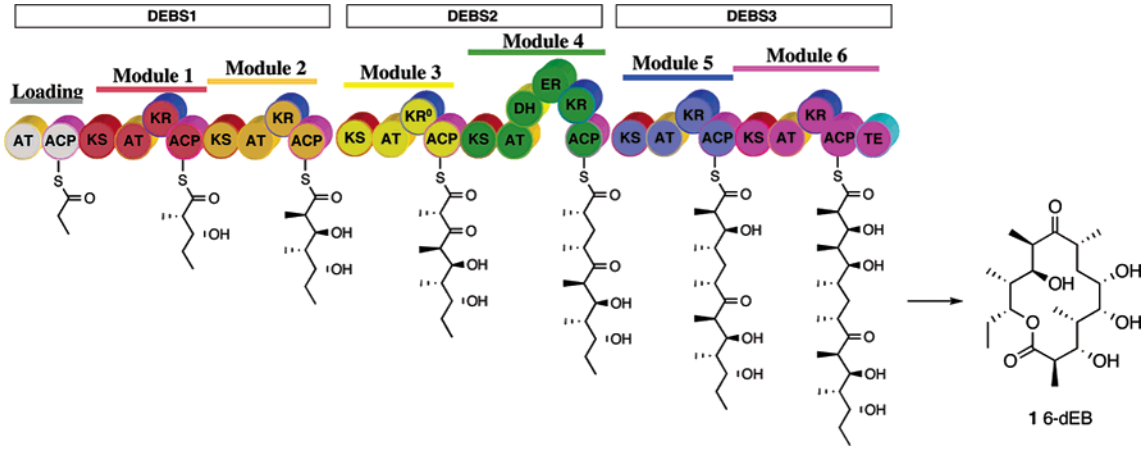
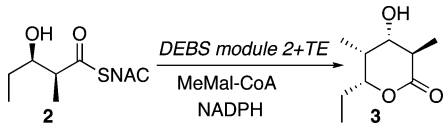


FIGURE 1: Modular organization of DEBS. In addition to the three core catalytic domains, a ketosynthase (KS), an acyl transferase (AT), and an acyl carrier protein (ACP) domain, individual modules can carry a variable combination of auxiliary keto reductase (KR), dehydratase (DH), and enoylreductase (ER) domains. A loading didomain primes module 1 with propionyl-CoA, and a thioesterase (TE) domain cyclizes the heptaketide to 6-dEB (**1**).

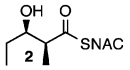
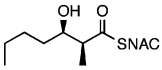
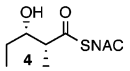
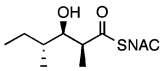
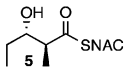
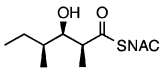
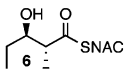
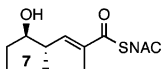
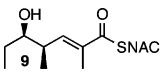
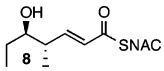
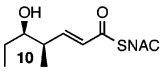
Scheme 1: Conversion of Diketide-SNAC **2** to Triketide Lactone **3** by DEBS Module 2+TE



acetylcysteamine analogue of the natural diketide-SACP substrate of DEBS module 2, in the presence of NADPH gives an acyclic triketide that is released from the enzyme by thioesterase-catalyzed cyclization to yield triketide lactone **3** (Scheme 1). Steady-state kinetic analysis gave a turnover number (k_{cat}) of 1.6 min^{-1} and a catalytic efficiency or specificity constant (k_{cat}/K_m) of 0.36 $mmol^{-1}$ min^{-1} for diketide **2** (*8*). Examination of a variety of diketide analogues of **2** revealed that variations in substitution pattern and substrate chain length had relatively modest effects on the relative k_{cat}/K_m (Table 1) (*8*, *9*). By contrast, module 2+TE exhibited a striking stereochemical specificity, with the enantiomeric syn-(2*R*,3*S*)-diketide **4** being converted to the corresponding triketide lactone at ~10% of the rate of **2** and with a 100-fold diminished k_{cat}/K_m, while neither of the two anti diastereomers, diketides **5** and **6**, was processed at all (*9*). Examination of a series of unsaturated triketides of varying stereochemistry and degree of methyl substitution revealed analogous trends for methyl substitution but opposite stereochemical preferences (Table 1) (*10*). Thus, the anti-substituted unsaturated triketide **7** and its 2-desmethyl analogue **8** were each processed efficiently, with **7** displaying a k_{cat}/K_m 1.5-fold greater than that of natural diketide **2**, while neither of the syn-triketides, **9** or **10**, was converted to the corresponding tetraketide products by DEBS module 2+TE.[2]

Processing of the natural diketide, as well as substrate analogues, by DEBS module 2+TE involves sequential condensation, reduction, and lactonization catalyzed in turn by the KS, KR, and TE domains, respectively (Figure 2). In principle, each of these biochemical steps might contribute to the observed k_{cat}. On the other hand, the value of k_{cat}/K_m can only be a function of those kinetic events up to and including the first irreversible step (*11*). Since the decarboxylative condensation reaction catalyzed by the KS domain is essentially irreversible, the observed k_{cat}/K_m for a given substrate necessarily reflects the intrinsic specificity of only the KS domain, and is therefore not influenced by any of the downstream domains of the PKS module. For the KS-catalyzed reaction, reversible binding of the polyketide substrate is followed by two consecutive chemical steps: the formation of a covalent acyl−enzyme intermediate attached

Table 1: Specificity of DEBS Module 2+TE for Representative Diketide- and Triketide-SNAC Substrates

Substrate	k_{cat}/K_m (rel)[a]	Substrate	k_{cat}/K_m (rel)[a]
 **2**	100[b]		300[b]
 **4**	1[b]		80[d]
 **5**	0[b]		550[d]
 **6**	0[b]		
 **7**	150[c]	 **9**	0[c]
 **8**	20[c]	 **10**	0[c]

[a] Because studies carried out at different times with different preparations of DEBS module 2+TE have yielded small variations in steady-state kinetic parameters for natural diketide **2**, all values of k_{cat}/K_m(rel) listed here have been normalized to 100 for **2**. [b] From ref *9*. k_{cat} > 4.6 min^{-1}. K_m(**2**) > 3.2 mM. k_{cat}/K_m = 0.75 $mmol^{-1}$ min^{-1}. [c] From ref *10*. k_{cat} = 1.2 min^{-1}. K_m(**2**) = 5.0 mM. k_{cat}/K_m = 0.24 $mmol^{-1}$ min^{-1}. The relative k_{cat}/K_m values for **7** and **8** are based on competitive incubations (cf. Figure 10; ref *10*). On the basis of direct determination of the k_{cat}/K_m for **7**, the relative k_{cat}/K_m compared to that of **2** is 120. [d] From ref *8*. k_{cat} = 1.56 min^{-1}. K_m(**2**) = 4.4 mM. k_{cat}/K_m = 0.36 $mmol^{-1}$ min^{-1}.

[2] By contrast, both anti-triketide **7** and syn-triketide **9** are processed by DEBS module 3+TE with equal catalytic efficiency, but at rates that are ~10% of those for processing of **7** by module 2+TE (*10*).

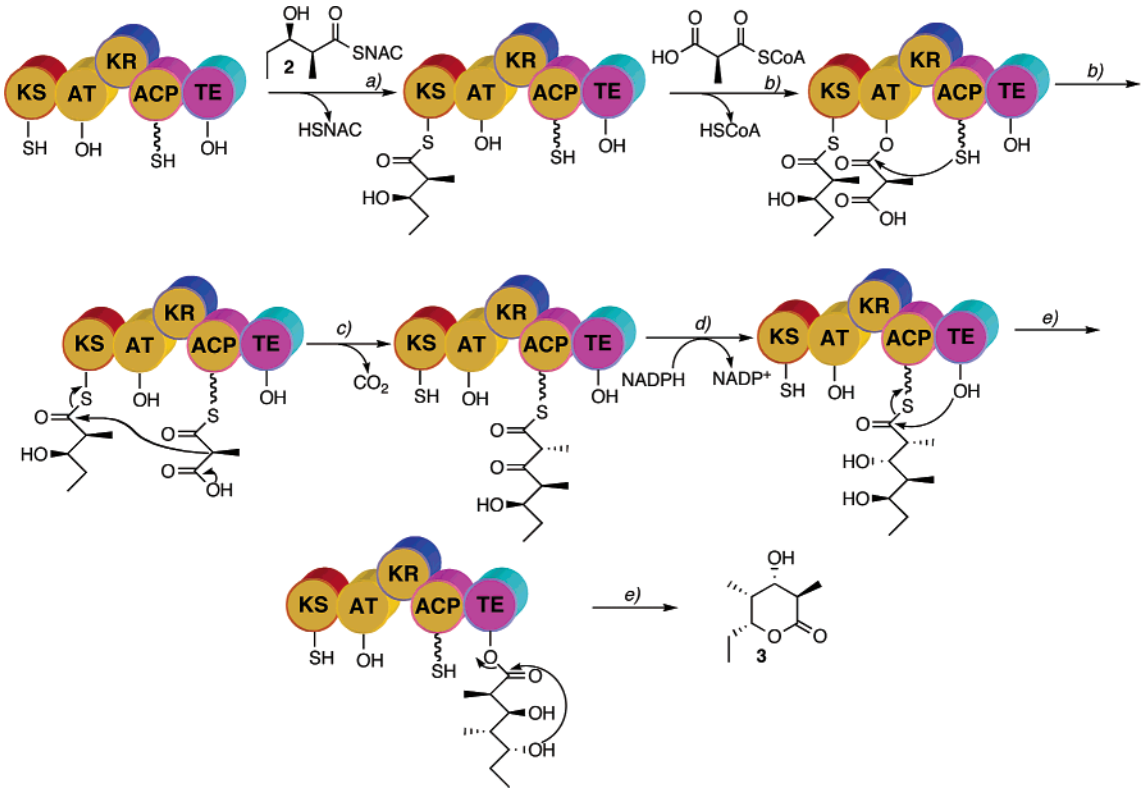


FIGURE 2: Chain elongation cycle catalyzed by DEBS module 2+TE. (a) The KS domain is acylated with a 2-methyl-3-hydroxypentanoyl unit from diketide **2**. (b) The ACP domain is primed with a methylmalonyl extender unit derived from its CoA derivative, in a reaction catalyzed by the AT domain. (c) Condensation then takes place in the active site of the KS domain with the release of carbon dioxide. (d) The KR domain reduces the β-ketoacyl-SACP thioester. (e) The TE domain catalyzes lactone formation with release of the final product.

to the active site Cys thiol and subsequent decarboxylative condensation of this acylthioester with the methylmalonyl-SACP, resulting in formation of a β-ketoacyl-SACP product. In principle, either or both of these two chemical steps might contribute to the observed substrate specificity of the KS domain. The ability of the decarboxylative condensation to influence the observed k_{cat}/K_m necessarily depends on whether prior formation of the acyl−enzyme intermediate is kinetically reversible. We report below a series of experiments using recombinant DEBS module 2 that are designed to probe both the intrinsic substrate selectivity and the reversibility of the KS-catalyzed formation of the acyl−enzyme intermediate.

EXPERIMENTAL PROCEDURES

Materials and Methods. Phosphorimaging was carried out using a Bio-Rad GS363 Molecular Imager System. Liquid scintillation counting was performed on a Beckman LS6500 liquid scintillation counter in 10 mL of Opti-Fluor scintillation cocktail. Radio-HPLC was carried out on a Rainin HPLC system equipped with a dual HPXL solvent delivery system and a Packard Radiomatic Flo-OneB detection system. ^{1}H NMR (300 MHz) utilized a Bruker Avance AM 300 spectrometer. (2*S*,3*R*)-[1-^{14}C]-2-Methyl-3-hydroxypentanoic acid *N*-acetylcysteamine thioester ([1-^{14}C]**2**, 55.0 mCi/mmol) was prepared by custom synthesis by Amersham Pharmacia Biotech. DNA manipulations were carried out via standard procedures (*12*). Diketide-SNAC derivatives **2**, **4**, and **5** and unsaturated triketides **7**−**10** were synthesized as previously described (*10*, *13*, *14*). Tris(2-carboxyethyl)-phosphine hydrochloride (TCEP), cystamine dihydrochloride, and [1-^{14}C]acetic anhydride were purchased from Sigma. Isopropyl thio-D-galactopyranoside (IPTG) was from Gibco. All other chemicals were purchased from Sigma-Aldrich. Nickel−nitriloacetic acid (Ni−NTA) resin was obtained from Qiagen. PD columns were from Amersham Pharmacia Biotech, and Centriprep-50 concentrators (molecular mass cutoff of 50 kDa) were from Amicon. DEBS module 2+TE was expressed in *E. coli*, purified, and assayed as previously described (*9*, *10*). Expression plasmid pPK22, a derivative of pET28a(+) harboring the DNA for DEBS module 2 with the natural C-terminal peptide linker or docking domain and carrying both N- and C-terminal His_6 tags (*15*), was a gift from P. Kumar. Protein concentrations determined by the Bradford assay (*16*) using bovine serum albumin (BSA) as a standard exhibited standard deviations of 5% for samples measured at different times. Kinetic data were analyzed by direct fitting to the relevant kinetic equations using the KaleidaGraph data analysis software (Synergy Software).

Expression and Purification of DEBS Module 2. Plasmid pPK22 was used to transform *E. coli* BAP1, an expression host derived from *E. coli* BL21(DE3) harboring the *sfp* gene encoding the surfactin acyl carrier protein synthase, which provides for post-translational phosphopantetheinylation of the ACP domain of module 2 (*17*). A 1 L culture was grown at 37 °C in LB medium supplemented with 50 g/mL kanamycin to an OD_{600} of 0.6. Protein expression was induced by addition of 1 mM IPTG, and the incubation was continued for 16 h at 25 °C. All protein purification procedures were carried out at 4 °C. The cells were harvested by centrifugation at 4200*g* and resuspended in lysis buffer [50 mM sodium phosphate (pH 8.0), 300 mM sodium

chloride, 10 mM imidazole, 10 mM β-mercaptoethanol, 1.5 mM benzamidine, pepstatin (2 mg/L), leupeptin (2 mg/L), and 20% (v/v) glycerol]. The resuspended cells were disrupted by being passed (four times) through a French press at 14 000 psi, and nucleic acids were precipitated with polyethylenimine (0.15%). The lysate was centrifuged at 40000*g*, and the supernatant was mixed with 10 mL of previously equilibrated Ni−NTA resin. The resulting slurry was stirred for 1 h before being loaded onto a capped Bio-Rad column (1 in. inside diameter). The column was washed with 10 bed volumes of 40 mM imidazole in 50 mM sodium phosphate buffer (pH 8.0) containing 300 mM sodium chloride and 20% (v/v/) glycerol, after which module 2 was eluted using a linear gradient of 40 to 250 mM imidazole in 10 bed volumes in the same buffer. Pooled fractions containing DEBS module 2 (90% pure as determined by SDS−PAGE) were concentrated and exchanged into storage buffer [100 mM sodium phosphate (pH 7.2), 1 mM EDTA, 1 mM TCEP, and 20% (v/v) glycerol] using a PD-10 column and then further concentrated with a Centriprep 50 unit before being flash-frozen in liquid nitrogen and stored at −80 °C.

Acylation of DEBS Module 2. Reactions were set up in 100 μL of phosphate incubation buffer [100 mM sodium phosphate buffer (pH 7.2) containing 1 mM EDTA, 1 mM TCEP, and 20% (v/v) glycerol]. The concentration of DEBS module 2 was adjusted to 12 μM, while the concentration of [1-^{14}C]diketide **2** (2.85 mCi/mmol) was varied from 0.3 to 4.8 mM. The concentration of DMSO, which was required to dissolve the diketide, was adjusted to 8% (v/v) for all incubations, which were carried out at 22 °C. Aliquots of 15 μL were withdrawn at periodic intervals from 10 to 360 s and the reactions quenched with 1 mL of ice-cold acetone together with 0.2 mg of BSA. The pellets were collected by centrifugation, and washed repeatedly with portions of cold 50% trichloroacetic acid until there was no detectable ^{14}C activity in the washings. The pellet was then dissolved in 200 μL of 8.0 M guanidinium·HCl and the covalently bound radioactivity quantitated by liquid scintillation counting (LSC). Control incubations contained all components except the module 2 protein. Each data point was determined at least three times.

Competitive Acylation of DEBS Module 2. Competitive acylation reactions of DEBS module 2 were set up and carried out as described above, using a mixture of 1.2 mM [1-^{14}C]**2** and 1.2 mM diketide **4** or **5** or triketides **7**−**10**.

Cerulenin Inhibition of Acylation of Module 2 by Diketide 2. Cerulenin [(2*S*,3*R*)-2,3-epoxy-4-oxo-7,10-dodecadienoylamide] (0.11−1.12 mM) was incubated with 9.3 μM DEBS module 2 at 22 °C in 50 μL of phosphate incubation buffer. Aliquots of 10 μL were withdrawn at 0, 2, 4, 8, and 20 min and mixed with 1 μL of 30 mM [1-^{14}C]diketide **2** (2.85 mCi/mmol) in DMSO. After incubation for 2 min at 22 °C, the reaction was quenched with 1 mL of cold acetone together with 0.2 mg of BSA. The pelleted protein was washed and analyzed as described above. Blank controls lacked the module 2 protein, and each data point was an average of at least three experiments.

N-[1′-^{14}C-acetyl]Cysteamine. To a 0.5 mL aqueous solution of 62 mg (0.27 mmol) of cystamine dihydrochloride, adjusted to pH 8.2 with saturated aqueous $NaHCO_3$, was added 0.123 mmol (1 mCi) of [1-^{14}C]acetic anhydride in 45 μL of dry THF with cooling in an ice/water bath, followed by addition of 11.7 μL (0.123 mmol) of unlabeled acetic anhydride in 80 μL of dry THF. The mixture was stirred for 1 h at 0 °C, then saturated with solid NaCl, and extracted with five 5 mL portions of methylene chloride. The pooled organic layers were dried over sodium sulfate, and the solvent was removed *in vacuo*. The resulting *N*,*N*′-[1′-^{14}C-*acetyl*]-diacetylcystamine was dissolved in 200 μL of DMSO and reduced by treatment with 232 mg (0.8 mmol) of TCEP hydrochloride in 4 mL of water for 3.0 h at 22 °C. The resulting *N*-[1′-^{14}C-*acetyl*]cysteamine was purified by HPLC using a Phenomenex C4 reverse phase column (250 mm × 10 mm) using a gradient over 30 min of 0 to 40% solvent B (95% acetonitrile and 5% water) in solvent A (2% acetonitrile and 98% water). The concentration of *N*-[1′-^{14}C-*acetyl*]-cysteamine (t_R = 8.9 min) was quantified by HPLC comparison to standard *N*-acetylcysteamine of a known concentration, and the specific activity was determined by LSC to be 1.12 mCi/mmol.

Module 2-Catalyzed Thioester Exchange of Diketide-SNAC 2 and Analogues with N-[1′-^{14}C-acetyl]Cysteamine. Thioester exchange reactions were carried out at 30 °C for 90 min in 32 μL of 100 mM phosphate incubation buffer (pH 7.2) in the presence of variable concentrations of diketide-SNAC **2** (1−8 mM), *N*-[1′-^{14}C-*acetyl*]cysteamine (1−8 mM), and 8% (v/v) DMSO. Control reactions included all the components except the module 2 protein. The reactions were quenched by the addition of 10 μL of 1 M HCl. The resulting *N*-[1′-^{14}C-*acetyl*]diketide-SNAC **2** (t_R = 12.0 min) was separated from *N*-[1′-^{14}C-*acetyl*]cysteamine (t_R = 5.7 min) by reverse phase HPLC on a Phenomenex Hydro-RP analytical column (250 mm × 4.6 mm) using a gradient over 15 min of 10 to 50% solvent B in solvent A, and the purified **2** was analyzed by LSC. Thioester exchange reactions with diketide-SNAC analogues **4** and **5** and triketides **7**−**10** (2 mM) were carried out in an analogous manner in 150 μL of 100 mM phosphate incubation buffer (pH 7.2) containing 4.8 μM module 2 and 2 mM *N*-[1′-^{14}C-*acetyl*]cysteamine, with 8% (v/v) DMSO. Aliquots of 25 μL were withdrawn at periodic intervals from 20 to 360 min and the reactions quenched with 5 μL of 1 M HCl. The labeled components were separated and purified by reverse phase HPLC, as described above, and the activity of the recovered diketide or triketide was determined by LSC.

Module 2-Catalyzed Thioester Exchange of Diketide-SNAC 2 and CoASH. Thioester exchange reactions were carried out at 30 °C for 30 min in 30 μL of 100 mM phosphate incubation buffer (pH 7.2) containing 8% (v/v) DMSO, variable concentrations of [1-^{14}C]diketide **2** (2.85 mCi/mmol, 1−8 mM), and fixed concentrations of CoASH (4 and 6 mM). The reactions were quenched by the addition of 10 μL of 1 M HCl and analyzed by reverse phase HPLC on a Phenomenex Hydro-RP analytical column (250 mm × 4.6 mm) using a gradient over 15 min of 10−40% solvent B in solvent C [2% acetonitrile in 20 mM sodium phosphate (pH 2.9)] (**2**-SCoA, t_R = 8.5 min; **2**, t_R = 13.4 min) with monitoring by radiodetection.

RESULTS

Kinetics and Stoichiometry of Acyl−Enzyme Adduct Formation. We have previously reported that treating the bimodular PKS subunit DEBS1 with [1-^{14}C]diketide **2** results

in exclusive and specific acylation of the active site Cys of the KS domain of module 2 (KS2), with the predicted 1:1 stoichiometry (*18*). This acylation reaction can be blocked by cerulenin (*18*), a well-established inhibitor of fatty acid (*19*) and polyketide biosynthesis that is known to act by covalently modifying the KS active site cysteine (*3*). The catalytic competence of the acyl−enzyme species for polyketide chain elongation was confirmed by treatment of [^{14}C]diketide-acylated DEBS1 with methylmalonyl-CoA and NADPH, which resulted in recovery of >50% of the ^{14}C activity in triketide lactone **3** (*4*). Acylation by [^{14}C]**2** of a wide range of recombinant PKS modules derived from the erythromycin (DEBS), picromycin (PICS), and rifamycin (RIFS) synthases has in fact been used as a sensitive and specific probe for the presence of a functional KS domain (*20*).

To investigate the biochemical basis for the observed k_{cat}/K_m of diketide **2** and its analogues, we began by examining the effect of substrate structure on the rate of acylation of the KS domain of DEBS module 2. Recombinant DEBS module 2, purified from *E. coli* harboring the *sfp* gene product to ensure post-translational phosphopantetheinylation of the ACP2 domain, was incubated at 22 °C with variable concentrations of [1-^{14}C]diketide **2**. Aliquots of the reaction mixture were withdrawn after periodic intervals up to a total incubation time of 6.0 min and the reactions quenched with acetone. The pelleted protein was analyzed by liquid scintillation counting (Figure 3). Control experiments in which the precipitated protein was subjected to separation by 5% SDS−PAGE and visualized by both Coomassie Blue and phosphorimaging confirmed that only the protein band at 158 kDa (M_r) was covalently labeled by [1-^{14}C]**2** (data not shown). The acylation data were analyzed on the basis of the kinetic scheme shown in Scheme 2, in which K_S represents the dissociation constant of the enzyme−diketide−SNAC complex and k_2 is the first-order rate constant for acylation of the active site Cys of the KS domain. Acylation by diketide-SNAC followed the expected exponential kinetics (*21*), with a k_2 of 5.8 ± 2.6 min^{-1} and a K_S of 3.5 ± 2.8 mM, as well as a limiting stoichiometry of ∼0.8:1 of diketide covalently bound to protein. The specificity of the acylation was confirmed by the observation that both the apparent rate and the end point of the acylation of module 2 by diketide could be reduced in a time-dependent (k_{inact} = 0.24 min^{-1}) and concentration-dependent (K_i = 1.2 mM) manner by preincubation with the KS inactivator cerulenin.

We next examined the ability of analogues of natural diketide **2** to acylate the KS domain. Rather than prepare each analogue in labeled form, we carried out competitive incubations with DEBS module 2 in the presence of [1-^{14}C]-**2** and six representative diketide- and triketide-SNAC analogues and measured the relative ability of each analogue to suppress acylation by labeled **2** (Figure 4). The syn-(2*R*,3*S*)-diketide **4** had no measurable effect on either the rate or end point of acylation by labeled **2**, consistent with the k_{cat}/K_m of **4** being 100-fold lower than that of **2** (*9*). Similarly, triketide **8**, with a k_{cat}/K_m that is 20% of that of diketide **2** (*10*), did not measurably attenuate the acylation of module 2 by labeled **2**. The anti-(2*S*,3*S*)-diketide **5**, which is not converted to product at all by DEBS module 2+TE (*9*), also failed to suppress acylation by syn-(2*S*,3*R*)-diketide **2**. On the other hand, triketide **7**, which we have previously

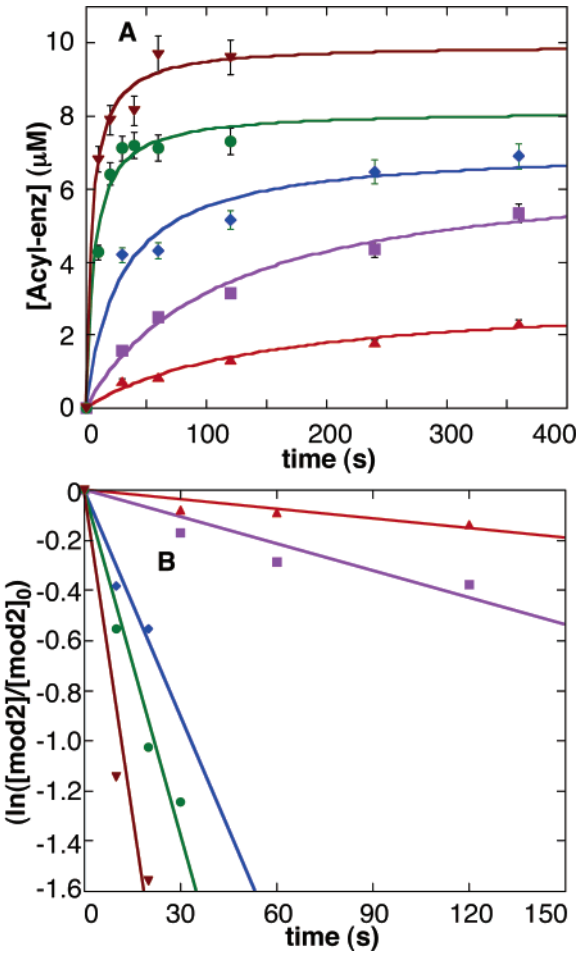
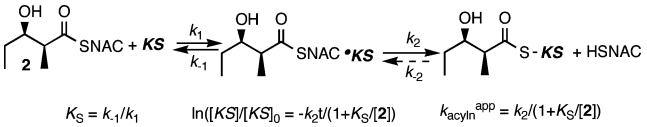


FIGURE 3: Acylation of DEBS module 2 by [1-^{14}C]diketide-SNAC **2**. Variable concentrations of [1-^{14}C]**2** (0.3 mM, red triangles; 0.6 mM, pink squares; 1.2 mM, blue diamonds; 2.4 mM, green circles; and 4.8 mM, red inverted triangles) were incubated with 12 M module 2. Reaction conditions are described in Experimental Procedures. (A) Time course of formation of the acyl−enzyme intermediate. (B) Natural log of the fraction of residual module 2 vs time. The slope of each line corresponds to $-k_{acyln}^{app}$ as defined in Scheme 2.

Scheme 2: Kinetics of Acylation of the KS Domain of DEBS Module 2 by Diketide-SNAC **2**



shown to be an excellent substrate for module 2+TE [relative k_{cat}/K_m that is 1.5 times greater than that of **2** (*10*)], inhibited acylation of module 2 by diketide **2** by ∼40%. Unexpectedly, both syn-triketides **9** and **10**, neither of which is converted to product by DEBS module 2+TE (*10*), each inhibited acylation of module 2 by diketide **2** by ∼40%.

Reversibility of Acyl−Enzyme Adduct Formation. To examine the reversibility of acyl−enzyme adduct formation, we carried out isotope exchange experiments with DEBS module 2. A mixture of unlabeled diketide-SNAC **2** and *N*-[1′-^{14}C-*acetyl*]cysteamine ([^{14}C]HSNAC) was incubated with module 2, and the appearance of radioactivity in the recovered diketide-SNAC **2** was measured (Scheme 3). Using a series of fixed concentrations of **2** in the presence of variable concentrations of [^{14}C]HSNAC, the data were fit

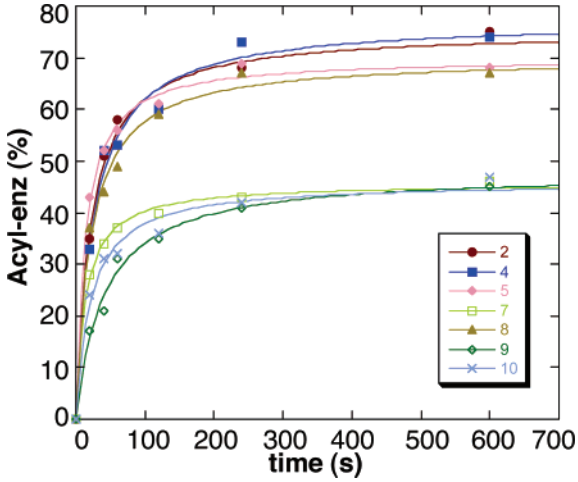
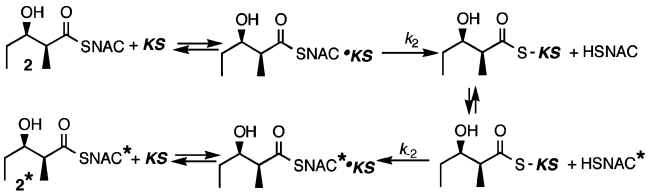


FIGURE 4: Competitive acylation. Each analogue (1.2 mM), mixed with 1.2 mM [1-^{14}C]diketide **2**, was incubated with 12 μM DEBS module 2. Aliquots of each incubation mixture were withdrawn at various time points up to a total incubation of 10 min and the reactions quenched with excess acetone, and the pelleted, labeled protein was analyzed by LSC. The data shown for **2** are for 1.2 mM [1-^{14}C]**2** alone, without added competitor. In a control experiment, coincubation of 1.2 mM unlabeled **2** with 1.2 mM [1-^{14}C]**2** reduced the observed level of covalent radioactive labeling of module 2 by the expected ~50% (data not shown).

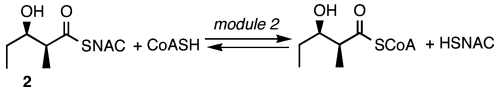
Scheme 3: Kinetics of Thioester Exchange between Diketide-SNAC **2** and [^{14}C]HSNAC Catalyzed by the KS Domain of DEBS Module 2



1) $V_{exch} = k_{exch}[\mathbf{2}][HSNAC]/\{(K_m^{\mathbf{2}} \cdot K_m^{HSNAC^*}) + (K_m^{\mathbf{2}} \cdot [\mathbf{2}]) + (K_m^{HSNAC^*} \cdot [\mathbf{HSNAC^*}])\}$

2) $k_{exch} = k_2 k_{-2}/(k_2 + k_{-2})$

Scheme 4: Thioester Exchange between Diketide-SNAC **2** and CoASH Catalyzed by the KS Domain of DEBS Module 2



to the standard Michaelis−Menten expression (Scheme 3, eq 1) to give a rate constant for exchange (k_{exch}) of 0.15 ± 0.06 min^{-1}, with a K_m for HSNAC of 5.7 ± 4.9 mM and a K_m for **2** of 5.3 ± 0.9 mM. Notably, as established by direct HPLC comparison with a synthetic sample, there was no detectable formation of any of the corresponding hydrolysis product, (2*S*,3*R*)-2-methyl-3-hydroxypentanoic acid, which would have resulted from the competing reaction of the acyl−enzyme intermediate with water. By contrast, the acyl−enzyme intermediate could also be intercepted by coenzyme A (Scheme 4). Thus, when coenzyme A was substituted for HSNAC as the nucleophile, DEBS module 2 catalyzed thioester exchange with diketide-SNAC **2** to give diketide-SCoA (**2**-ScoA) at a rate of 0.044 ± 0.002 min^{-1} with a K_m for CoASH of 3.5 ± 0.4 mM.

Finally, we also determined the rates of diketide thioester−HSNAC exchange for the same panel of diketide and

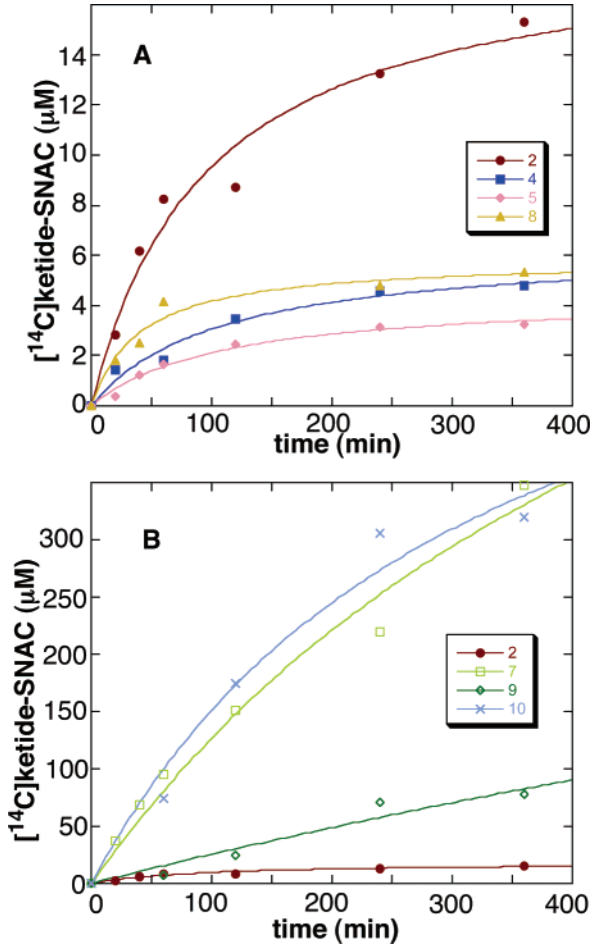


FIGURE 5: Thioester exchange between 2 mM diketide- and triketide-SNAC analogues and 2 mM [^{14}C]HSNAC catalyzed by the KS domain of DEBS module **2** (6.2 μM). Time course of incorporation of [^{14}C]SNAC into diketide- and triketide-SNAC analogues. Data are corrected for non-enzyme-catalyzed background thioester exchange. The calculated k_{exch}^{obs} for each exchange reaction is listed in Table 2: (A) analogues **2**, **4**, **5**, and **8** and (B) analogues **2**, **7**, **9**, and **10**.

Table 2: Rate of Thioester Exchange between Diketide- and Triketide-SNAC Analogues and [^{14}C]HSNAC Catalyzed by the KS Domain of DEBS Module 2[a]

	2	**4**	**5**	**7**	**8**	**9**	**10**
k_{exch}^{obs} (min^{-1})	0.071	0.015	0.011	0.54	0.034	0.14	0.70
k_{exch}^{rel}	1	0.21	0.16	7.6	0.48	1.9	9.8

[a] Substrate concentration of 2 mM; [^{14}C]HSNAC concentration of 2 mM.

triketide analogues (Figure 5 and Table 2). DEBS module 2-catalyzed transthioesterification of diketides **4** and **5** by [^{14}C]HSNAC took place at rates that were 20 and 16% of that of diketide **2**, respectively, while anti-triketide **8** underwent isotope exchange at a rate that was 50% of that of natural diketide **2**. Intriguingly, triketides **9**, **7**, and **10** exhibited relative 1.9-, 7.6-, and 9.8-fold enhancements, respectively, in their rates of thioester exchange compared to that of **2**, although neither **9** nor **10** is actually converted to product by module 2+TE.

DISCUSSION

The programming of the complex series of reactions comprising polyketide biosynthesis is the consequence of a hierarchy of molecular controls.

(1) At the organizational level, the number and order of the individual biochemical reactions depend on the specific modular composition of each PKS. Thus, the number of chain elongation steps is a direct function of the number of modules (*22*−*26*), while the individual biochemical transformations that accompany each chain elongation event are a consequence of the specific set of domains harbored within each module (*1*, *4*). Among these domains, the acyltransferase (AT) domains play a special role as a gatekeeper by exercising strict specificity over the choice of malonyl-, methylmalonyl-, or ethylmalonyl-CoA chain elongation substrate (*4*, *27*, *28*). Following the KS-catalyzed chain elongation reaction, modification of the resultant β-ketoacyl-ACP is determined by the precise mix of ketoreductase (KR), dehydratase (DH), and enoylreductase (ER) domains, as well as the presence of other, less common modifying activities such as the SAM-dependent methyl transferases found in the epothilone (*29*, *30*) hybrid NRPS−PKS system. In some modules, an additional epimerase is responsible for the generation of chain elongation products with L-methyl stereochemistry (*31*, *32*). Although this epimerase activity appears to be associated with the KS domain, the location and composition of the epimerase active site are still unknown, and even the precise timing of the epimerization events remains an open question (*33*, *34*).[3] The particular set of reactions catalyzed by each module is controlled not only by the specific suite of domains that are present but also by a still poorly understood competition between the processing of each intermediate by its cognate domain and premature transfer of the polyketide product to the KS domain of the downstream module (*26*, *35*).[4] Occasionally, the chain elongation product may even undergo back transfer to the KS domain of the same module, a phenomenon which has been termed stuttering (*36*, *37*) and which is more typical of iterative PKS enzymes.

(2) An essential feature of the programming of polyketide biosynthesis involves the accurate transfer of each polyketide chain elongation intermediate from the producing module to the proper downstream module. When both modules are part of the same polypeptide, transfer from the donor ACP domain to the acceptor KS domain involves straightforward intraprotein translocation of the growing acyl chain. On the other hand, when the two modules are in separate proteins, correct transfer to the proper downstream module involves protein−protein molecular recognition. For the DEBS PKS, for example, this interprotein module−module communication is mediated in large part by specific pairing of relatively short, 50−100-amino acid peptide segments, which have been termed peptide linkers or, more precisely, docking domains, located at the C-terminus and N-terminus of the donor and acceptor modules, respectively, that transiently associate through apparent coil−coil interactions (*15*, *38*−*40*). Similar requirements have been reported for productive polyketide chain transfer between PICS modules 5 and 6 (*41*).

(3) The KS domain of each acceptor module, while having a broad substrate tolerance, can also possess significant intrinsic substrate specificity. Such substrate preferences are not ordinarily apparent in native PKS systems, in which each module only encounters its natural substrate, which it acquires from the proper upstream donor module. Individual modules can be challenged, however, with a wide variety of structural variants of their natural polyketide substrates, whether by (a) the use of chimeric modules made up of some combination of inactivated, deleted, altered, or added domains, (b) engineered substrate transfer between pairs of modules that normally do not communicate, (c) precursor-directed biosynthesis, or (d) *in vitro* incubations with recombinant PKS modules. Determining the intrinsic substrate specificity of individual PKS modules and understanding its biochemical basis are therefore critical to the successful design of hybrid PKS systems and for the application to combinatorial biosynthesis of new natural products.

The substrate specificity of individual polyketide synthase modules has been extensively probed using a wide variety of diketide- and triketide-SNAC derivatives that are structural and stereochemical analogues of natural polyketide chain elongation intermediates (*8*−*10*, *41*). In each case, the k_{cat}/K_m, or specificity constant, for catalysis of a single round of chain elongation, reductive modification, and release of the resulting extended polyketide by TE-catalyzed cyclization or hydrolysis reflects the intrinsic specificity of only the KS domain of the specific module. Importantly, it has been found that although the corresponding diketide-SACP substrates exhibit ca. 1000-fold increases in the observed values of k_{cat}/K_m, compared to those of the diketide-SNAC derivatives, the use of the diketide-SACP analogues has no significant effect on the relative values of k_{cat}/K_m for the various diketide derivatives (*38*). This observation validates the use of the more conveniently prepared -SNAC thioesters in probing the biochemical basis of ketosynthase specificity.

The KS-catalyzed polyketide chain elongation reaction involves two discrete chemical steps (Figure 2). Reversible binding of the polyketide substrate is followed by covalent attachment of the electrophilic component of the substrate to an active site cysteine residue, with displacement of the natural -SACP or analogue -SNAC leaving group. The resulting acyl−enzyme intermediate then undergoes nucleophilic attack by an enol or enolate generated by KS-catalyzed decarboxylation of a methylmalonyl or malonyl residue, attached to the ACP domain of the paired protein subunit of the homodimeric PKS module. Both crystallographic and site-directed mutagenesis studies have confirmed the role of the active site Cys and established that specific His and Lys residues both play essential roles in the decarboxylative condensation that breaks down the acyl−enzyme intermediate (*42*−*45*). The ketosynthase reaction therefore shares several important biochemical, mechanistic, and kinetic features with the reactions of the well-studied serine and

[3] Experiments with a hybrid of DEBS modules 1 and 2 have suggested that the KR1 domain controls methyl stereochemistry in the 2-methyl-3-hydroxy acylthioester product by selecting the corresponding epimerized diastereomer of the 3-ketoacylthioester produced by the KS1 domain (*33*). Subsequently, reported results with KR domains of modules 5 and 6 led to more ambiguous conclusions (*34*). This model also does not account for the epimerized L-methyl group in the 2-methyl-3-ketoacylthioester product of DEBS module 3, which lacks an active KR domain.

[4] Premature chain transfer has most often been observed in engineered hybrid modules constructed by swapping or addition of domains originating from homologous or heterologous PKS modules (*26*). In addition, silent DH domains, such as those found in several modules of the rapamycin PKS, might reflect the kinetics of domain skipping rather than the intrinsic lack of activity of the specific DH domain (*35*).

cysteine proteases and related esterases. Interestingly, detailed studies of serine proteases such as chymotrypsin have shown that overall substrate specificity is vested primarily in the formation of the acyl−enzyme intermediate, rather than in the subsequent hydrolytic breakdown step (*46*). Unlike the proteases and esterases, however, the formation of the acyl−enzyme intermediate of the KS reaction can be studied directly simply by omission of the nucleophilic (methyl)-malonyl-CoA substrate, without significant interference from competing hydrolytic breakdown (*47*). It therefore becomes possible to determine the first-order rate constant for formation of the acyl−enzyme intermediate (k_2) as well as the rate constant for the reverse reaction (k_{-2}).

The KS-catalyzed formation of the acyl−enzyme intermediate can be treated by the classical method of Kitz and Wilson, originally developed to analyze the covalent modification of the active site serine of acetylcholinesterase (*21*) (Scheme 2). Incubation of DEBS module 2 with [1-^{14}C]-diketide **2** allowed assignment of the first-order rate constant for acylation (k_2) of 5.8 ± 2.6 min^{-1}. Comparison with the k_{cat} of 1.6 min^{-1} for conversion of diketide **2** by module 2+TE to triketide lactone **3** confirms that the rate of acyl−enzyme adduct formation by module 2 alone is kinetically competent, assuming that the presence of the C-terminally appended TE domain does not significantly affect the activity of the KS domain. Similarly, the calculated dissociation constant (K_S) of 3.5 ± 2.8 mM for diketide **2** and module 2 compares favorably with the K_m of 4.4 mM previously determined for module 2+TE and **2**.[5] The effective equivalence of the diketide dissociation constant (K_S) and the Michaelis constant (K_m) suggests that $k_{-1} \gg k_2$, implying rapid equilibrium binding of the diketide substrate, consistent with the relatively modest values observed for the KS acylation rate constant (k_2).

By carrying out competitive acylations of DEBS module 2 using mixtures of [1-^{14}C]**2** and several diketide and triketide analogues, we hoped to determine the relative values of k_2/K_S by assessing the ability of each analogue to suppress both the rate and end point of formation of the labeled protein. The majority of the experimental results, however, illustrated in Figure 4, were at best of only qualitative significance, in large part due to the intrinsic insensitivity of the experimental method. Thus, coincubation of [1-^{14}C]**2** with an equal concentration of an analogue with an identical k_{cat}/K_m can only suppress the observed level of protein labeling by a factor of 2, while admixture with an analogue with a k_{cat}/K_m that is $^1/_5$ of that of natural diketide **2** would reduce the extent of labeling by only 10%, close to the experimental precision of the protein precipitation−LSC detection method that was used. Unfortunately, the limited solubility of the individual −SNAC esters makes it impractical to compensate for low relative values of k_{cat}/K_m with increases in substrate concentration, while the detection limits of phosphorimaging combined with the high molecular mass of DEBS module 2 (M_D = 158 kDa/subunit of the homodimer) do not allow significant reduction in the concentration of labeled diketide **2**. For example, triketide **7**, which has a k_{cat}/K_m value that is 1.5 times greater than that of **2**, effects a ∼40% reduction in the extent of protein labeling, while neither enantio-diketide **4**, with a k_{cat}/K_m that is 1% of that of [1-^{14}C]**2**, nor anti-diketide **5**, which is not converted to product at all by module 2+TE, has any experimentally significant effect on the observed labeling of module 2 by [1-^{14}C]**2**. Indeed, we have previously encountered a similar lack of dynamic sensitivity in competitive acylation experiments with the DEBS AT-loading domain (*48*). One surprising observation, however, was that both syn-triketides **9** and **10**, neither of which is converted to product by DEBS module 2+TE, each inhibited acylation of module 2 by diketide **2** by ∼40% (Figure 4). The latter results indicate that these apparent nonsubstrates are in fact capable of efficiently forming the corresponding acyl−enzyme derivatives, but that the latter species must be dead-end intermediates that do not undergo subsequent decarboxylative condensation with methylmalonyl-SACP (k_3**^{9}** and k_3**^{10}** ≅ 0).

The KS-catalyzed conversion of diketide-SNAC **2** to the β-ketoacyl-SACP triketide corresponds to a two-stage, double-displacement, or ping-pong type, mechanism. Having determined the rate constant for formation of the acyl−enzyme intermediate, we carried out equilibrium isotope exchange experiments with unlabeled diketide **2** and *N*-[1′-^{14}C-*acetyl*]cysteamine ([^{14}C]HSNAC). In these experiments, the use of DEBS module 2 instead of module 2+TE is essential in avoiding competing thioester exchange catalyzed by the appended TE domain, which can efficiently hydrolyze diketide- and triketide-SNAC substrates (*49*). The calculated rate constant for the appearance of the label in recovered, purified diketide (Scheme 3) was 0.15 ± 0.06 min^{-1}. This steady-state rate constant for thioester exchange (k_{exch}) is equal to $k_2k_{-2}/(k_2 + k_{-2})$. Since $k_{-2} \ll k_2$, the observed value of k_{exch} therefore corresponds to k_{-2}, the rate constant for the reversion of the acyl−enzyme intermediate with NACSH to diketide. The K_m measured for diketide **2** (5.3 ± 0.9 mM) is in good agreement with the K_S of 3.5 ± 2.8 mM determined by the protein acylation experiment as well as the K_m of 4.4 mM for turnover by module 2+TE. Module 2 also was able to catalyze thioester exchange between diketide-SNAC **2** and HSCoA, but at a rate 3-fold slower than that observed for HSNAC. In neither case did we observe competing hydrolysis of the diketide, indicating that water does not act as a surrogate nucleophile to attack the acyl−enzyme intermediate generated by DEBS module 2.

The observed module 2-catalyzed HSNAC−thioester exchange of diketide **4**, as well as of the anti-unsaturated triketides **7** and **8**, confirms the formation of the expected acyl−enzyme intermediate derived from each of these surrogate substrates. More surprising was the enzyme-catalyzed thioester exchange of anti-diketide **5** and the syn-unsaturated triketides **9** and **10**, none of which undergo chain elongation catalyzed by module 2+TE. The latter results confirm the formation of dead-end, acyl−enzyme intermediates derived from each of these substrate analogues. We have recently reported formation of dead-end acyl−enzyme intermediates derived from diketide-SNAC **2** and several homologous and heterologous PKS modules (*20*). The 2−10-fold enhanced rate of thioester exchange of the three triketides, **7**, **9**, and **10**, compared to that of natural diketide

[5] In a separate series of experiments, we also examined acylation of apo module 2, lacking the phosphopantetheinyl residue. Although apo module 2 underwent near-stoichiometric acylation by [1-^{14}C]**2**, not only was the apparent second-order rate constant for acylation (k_2/K_S) 0.056 mM^{-1} min^{-1}, ∼30-fold lower than that for holo module 2, but there was no evidence of saturation behavior up to 4.8 mM diketide.

2 is also significant, since it establishes that the rate of thioester exchange of diketide **2** is not kinetically limited by slow release of the *N*-acetylcysteamine leaving group from the enzyme following formation of the diketide acyl−enzyme intermediate.

Formation of the Acyl−Enzyme Intermediate Determines the Observed k_{cat}/K_m. As previously discussed, the k_{cat}/K_m observed for diketide and triketide substrates of PKS modules is solely a function of the KS domain. Determining which of the two chemical steps, formation of the acyl−enzyme intermediate or its subsequent conversion to the β-ketoacyl-SACP product, contributes to this specificity constant requires knowledge of whether formation of the acyl−enzyme intermediate is reversible or, more rigorously, determination of the relative values of k_{-2}, the rate constant for the reverse reaction, and k_3, the rate constant for the decarboxylative acylation. The protein acylation and thioester exchange experiments described above have provided values for k_2 and k_{-2}, respectively, as well as the value of K_S ($=k_{-1}/k_1$). The overall k_{cat} for conversion of diketide-SNAC **2** to triketide lactone **3** is 1.6 min^{-1}. Although the microscopic rate constants for the reactions catalyzed by the KR domain of module 2 and the appended TE domain are not known, from the measured value of k_2, it is evident that the rate constant (k_3) for the KS-catalyzed conversion of the diketide acyl−enzyme intermediate to β-ketoacyl-SACP must be at least 2 min^{-1}, or even greater if indeed either the downstream KR or TE domains contribute to the observed k_{cat}. Therefore, for natural diketide **2**, a $k_3/(k_{-2} + k_3)$ of 0.94, implying that formation of the acyl−enzyme intermediate, while technically reversible ($k_{-2} = 0.15$ min^{-1}), is effectively irreversible under steady-state conditions. The observed k_{cat}/K_m for diketide **2** therefore reflects overwhelmingly the intrinsic selectivity of the acyl−enzyme adduct-forming step. The PKS module, and specifically the KS domain, thus resembles serine proteases such as trypsin and chymotrypsin, whose intrinsic substrate specificities also reflect the selectivity of their acyl−enzyme adduct-forming biochemical step. The same considerations also apply to PKS substrate analogues with a k_{cat}/K_m that is 1% of that of diketide **2**. For example, for enantio-diketide **4**, the k_{cat} of 0.25 min^{-1} (*9*) sets a lower limit of 0.25 min^{-1} for k_3[4]. Since the thioester exchange of **4** has established k_{-2} to be 0.015 min^{-1}, the value of $k_3/(k_{-2} + k_3)$ is again essentially 1, requiring thereby that acyl−enzyme adduct formation be effectively irreversible. Only for analogues such as **9** and **10**, which are not converted to product at all but are instead converted to dead-end acyl−enzyme intermediates ($k_3 = 0$ min^{-1}), is the apparent discrimination ultimately a function of a step other than acyl−enzyme adduct formation. Recent progress in determining the structures of KS domains (*45*) suggests that crystal structures might reveal differences in the active site conformations of productive and dead-end acyl−enzyme intermediates that could account for the striking differences in reactivity.

REFERENCES

1. 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.
2. Cortes, J., Haydock, S. F., Roberts, G. A., Bevitt, D. J., and Leadlay, P. F. (1990) An Unusually Large Multifunctional Polypeptide in the Erythromycin-Producing Polyketide Synthase of *Saccharopolyspora erythraea*, *Nature 348*, 176−178.
3. Pieper, R., Luo, G. L., Cane, D. E., and Khosla, C. (1995) Cell-free synthesis of polyketides by recombinant erythromycin polyketide synthases, *Nature 378*, 263−266.
4. 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.
5. Cane, D. E., and Walsh, C. T. (1999) The parallel and convergent universes of polyketide synthases and nonribosomal peptide synthetases, *Chem. Biol. 6*, R319−R325.
6. Walsh, C. T. (2004) Polyketide and nonribosomal peptide antibiotics: Modularity and versatility, *Science 303*, 1805−1810.
7. 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.
8. Kinoshita, K., Khosla, C., and Cane, D. E. (2003) Precursor-Directed Biosynthesis: Stereospecificity for Branched-Chain Diketides of the β-Ketoacyl-ACP Synthase Domain 2 of 6-Deoxyerythronolide B Synthase, *Helv. Chim. Acta 86*, 3889−3905.
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. Cane, D. E., Kudo, F., Kinoshita, K., and Khosla, C. (2002) Precursor-directed biosynthesis: Biochemical basis of the remarkable selectivity of the erythromycin polyketide synthase toward unsaturated triketides, *Chem. Biol. 9*, 131−142.
11. Northrop, D. B. (1998) On the Meaning of K_m and *V/K* in Enzyme Kinetics, *J. Chem. Educ. 75*, 1153−1157.
12. Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989) *Molecular Cloning, A Laboratory Manual*, 2nd ed., Cold Spring Harbor Laboratory Press, Plainview, NY.
13. Jacobsen, J. R., Hutchinson, C. R., Cane, D. E., and Khosla, C. (1997) Precursor-directed biosynthesis of erythromycin analogs by an engineered polyketide synthase, *Science 277*, 367−369.
14. Harris, R. C., Cutter, A. L., Weissman, K. J., Hanefeld, U., Timoney, M. C., and Staunton, J. (1998) Enantiospecific Synthesis of Analogs of the Diketide Intermediate of the Erythromycin Polyketide Synthase (PKS), *J. Chem. Res. 283* (Suppl.), 1230−1247.
15. Kumar, P., Li, Q., Cane, D. E., and Khosla, C. (2003) Intermodular communication in modular polyketide synthases: Structural and mutational analysis of linker mediated protein−protein recognition, *J. Am. Chem. Soc. 125*, 4097−4102.
16. Bradford, M. (1976) A Rapid and Sensitive Method for the Quantitation of Microgram Quantitites of Protein Utilizing the Principle of Protein-Dye Binding, *Anal. Biochem. 72*, 248−254.
17. Pfeifer, B. A., Admiraal, S. J., Gramajo, H., Cane, D. E., and Khosla, C. (2001) Biosynthesis of complex polyketides in a metabolically engineered strain of *E. coli*, *Science 291*, 1790−1792.
18. Tsukamoto, N., Chuck, J.-A., Luo, G., Kao, C. M., Khosla, C., and Cane, D. E. (1996) 6-Deoxyerythronolide B Synthase 1 Is Specifically Acylated by a Diketide Intermediate at the β-Ketoacyl Acyl Carrier Protein Synthase Domain of Module 2, *Biochemistry 35*, 15244−15248.
19. Kauppinen, S., Siggaard-Andersen, M., and von Wettstein-Knowles, P. (1988) β-Ketoacyl-ACP Synthase of *Escherichia coli*: Nucleotide Sequence of the *fabB* Gene and Identification of the Cerulenin Binding Residue, *Carlsberg Res. Commun. 53*, 357−370.
20. Watanabe, K., Wang Clay, C. C., Boddy Christopher, N., Cane David, E., and Khosla, C. (2003) Understanding substrate specificity of polyketide synthase modules by generating hybrid multimodular synthases, *J. Biol. Chem. 278*, 42020−42026.
21. Kitz, R., and Wilson, I. B. (1962) Esters of Methanosulfonic Acid as Irreversible Inhibitors of Acetylcholinesterase, *J. Biol. Chem. 237*, 3245−3249.
22. Kao, C. M., Luo, G., Katz, L., Cane, D. E., and Khosla, C. (1994) Engineered biosynthesis of a triketide lactone from an incomplete modular polyketide synthase, *J. Am. Chem. Soc. 116*, 11612−11613.
23. Kao, C. M., Luo, G., Katz, L., Cane, D. E., and Khosla, C. (1995) Manipulation of Macrolide Ring Size by Directed Mutagenesis of a Modular Polyketide Synthase, *J. Am. Chem. Soc. 117*, 9105−9106.
24. Cortes, J., Wiesmann, K. E. H., Roberts, G. A., Brown, M. J. B., Staunton, J., and Leadlay, P. F. (1995) Repositioning of a domain

in a modular polyketide synthase to promote specific chain cleavage, *Science 268*, 1487−1489.
25. Cao, C. M., Luo, G., Katz, L., Cane, D. E., and Khosla, C. (1996) Engineered Biosynthesis of Structurally Diverse Tetraketides by a Trimodular Polyketide Synthase, *J. Am. Chem. Soc 118*, 9184−9185.
26. Kao, C. M., McPherson, M., McDaniel, R. N., Fu, H., Cane, D. E., and Khosla, C. (1997) Gain of Function Mutagenesis of the Erythromycin Polyketide Synthase. 2. Engineered Biosynthesis of an Eight-Membered Tetraketide Lactone, *J. Am. Chem. Soc. 119*, 11339−11340.
27. Marsden, A. F. A., 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.
28. Lau, J., Fu, H., Cane, D. E., and Khosla, C. (1999) Dissecting the role of acyltransferase domains of modular polyketide synthases in the choice and stereochemical fate of extender units, *Biochemistry 38*, 1643−1651.
29. Molnar, I., Schupp, T., Ono, M., Zirkle, R. E., Milnamow, M., Nowak-Thompson, B., Engel, N., Toupet, C., Strattmann, A., Cyr, D. D., Gorlach, J., Mayo, J. M., Hu, A., Goff, S., Schmid, J., and Ligon, J. M. (2000) The biosynthetic gene cluster for the microtubule-stabilizing agents epothilones A and B from *Sorangium cellulosum* So ce90, *Chem. Biol. 7*, 97−109.
30. Tang, L., Shah, S., Chung, L., Carney, J., Katz, L., Khosla, C., and Julien, B. (2000) Cloning and Heterologous Expression of the Epothilone Gene Cluster, *Science 287*, 640−642.
31. Cane, D. E., Liang, T. C., Taylor, P. B., Chang, C., and Yang, C. C. (1986) Macrolide biosynthesis. 3. Stereochemistry of the chain-elongation steps of erythromycin biosynthesis, *J. Am. Chem. Soc. 108*, 4957−4964.
32. Weissman, K. J., Timoney, M. B., Grice, P., Hanefeld, U., Staunton, J., and Leadlay, P. F. (1997) The molecular basis of Celmer's rules: The stereochemistry of the condensation step in chain extension on the erythromycin polyketide synthase, *Biochemistry 36*, 13849−13855.
33. Holzbaur, I. E., Harris, R. C., Bycroft, M., Cortes, J., Bisang, C., Staunton, J., Rudd, B. A., and Leadlay, P. F. (1999) Molecular basis of Celmer's rules: The role of two ketoreductase domains in the control of chirality by the erythromycin modular polyketide synthase, *Chem. Biol. 6*, 189−195.
34. Holzbaur, I. E., Ranganathan, A., Thomas, I. P., Kearney, D. J., Reather, J. A., Rudd, B. A., Staunton, J., and Leadlay, P. F. (2001) Molecular basis of Celmer's rules: Role of the ketosynthase domain in epimerisation and demonstration that ketoreductase domains can have altered product specificity with unnatural substrates, *Chem. Biol. 8*, 329−340.
35. Aparicio, J. F., Molnar, I., Schwecke, T., Konig, A., Haydock, S. F., Khaw, L. E., Staunton, J., and Leadlay, P. F. (1996) Organization of the biosynthetic gene cluster for rapamycin in *Streptomyces hygroscopicus*: Analysis of the enzymatic domains in the modular polyketide synthase, *Gene 169*, 9−16.
36. Wilkinson, B., Foster, G., Rudd, B. A., Taylor, N. L., Blackaby, A. P., Sidebottom, P. J., Cooper, D. J., Dawson, M. J., Buss, A. D., Gaisser, S., Bohm, I. U., Rowe, C. J., Cortes, J., Leadlay, P. F., and Staunton, J. (2000) Novel octaketide macrolides related to 6-deoxyerythronolide B provide evidence for iterative operation of the erythromycin polyketide synthase, *Chem. Biol. 7*, 111−117.
37. Beck, B. J., Aldrich, C. C., Fecik, R. A., Reynolds, K. A., and Sherman, D. H. (2003) Iterative chain elongation by a pikromycin monomodular polyketide synthase, *J. Am. Chem. Soc. 125*, 4682−4683.
38. 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.
39. Tsuji, S. Y., Cane, D. E., and Khosla, C. (2001) Selective Protein−Protein Interactions Direct Channeling of Intermediates Between Polyketide Synthase Modules, *Biochemistry 40*, 2326−2331.
40. 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.
41. Beck, B. J., Aldrich, C. C., Fecik, R. A., Reynolds, K. A., and Sherman, D. H. (2003) Substrate recognition and channeling of monomodules from the pikromycin polyketide synthase, *J. Am. Chem. Soc. 125*, 12551−12557.
42. Witkowski, A., Joshi, A., Lindqvist, Y., and Smith, S. (1999) Conversion of a β-ketoacyl synthase to a malonyl decarboxylase by replacement of the active-site cysteine with glutamine, *Biochemistry 38*, 11643−11650.
43. Huang, W., Jia, J., Edwards, P., Dehesh, K., Schneider, G., and Lindqvist, Y. (1998) Crystal structure of β-ketoacyl-acyl carrier protein synthase II from *E. coli* reveals the molecular architecture of condensing enzymes, *EMBO J. 17*, 1183−1191.
44. Witkowski, A., Joshi, A. K., and Smith, S. (2002) Mechanism of the β-ketoacyl synthase reaction catalyzed by the animal fatty acid synthase, *Biochemistry 41*, 10877−10887.
45. Keatinge-Clay, A. T., Maltby, D. A., Medzihradszky, K. F., Khosla, C., and Stroud, R. M. (2004) An antibiotic factory caught in action, *Nat. Struct. Mol. Biol. 11*, 888−893.
46. Hedstrom, L., Perona, J. J., and Rutter, W. J. (1994) Converting Trypsin to Chymotrypsin: Residue 172 Is a Substrate Specificity Determinant, *Biochemistry 33*, 8757−8563.
47. Fastrez, J., and Fersht, A. R. (1973) Demonstration of the acyl-enzyme mechanism for the hydrolysis of peptides and anilides by chymotrypsin, *Biochemistry 12*, 2025−2034.
48. Lau, J., Cane, D. E., and Khosla, C. (2000) Substrate Specificity of the Loading Didomain of the Erythromycin Polyketide Synthase, *Biochemistry 39*, 10514−10520.
49. Gokhale, R. S., Hunziker, D., Cane, D. E., and Khosla, C. (1999) Mechanism and specificity of the terminal thioesterase domain from the erythromycin polyketide synthase, *Chem. Biol. 6*, 117−125.

BI048147G