6-Deoxyerythronolide B Synthase 1 Is Specifically Acylated by a Diketide Intermediate at the β-Ketoacyl-Acyl Carrier Protein Synthase Domain of Module 2†

Naohiro Tsukamoto,‡ Jo-Anne Chuck,‡ Guanglin Luo,‡ Camilla M. Kao,§ Chaitan Khosla,*,§ and David E. Cane*,‡

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

Received August 7, 1996⊗

ABSTRACT: We have used 6-deoxyerythronolide B synthase (DEBS) as a model system to investigate molecular recognition by a modular polyketide synthase (PKS). DEBS consists of three proteins (DEBS1, -2, and -3) that biosynthesize the polyketide skeleton of the antibiotic erythromycin from propionyl-CoA and methylmalonyl-CoA. Active sites within these multifunctional proteins are organized into biosynthetic "modules", each of which catalyzes a discrete round of polyketide chain elongation and adjusts the appropriate level of β-ketoacylthioester reduction. Using DEBS1, we demonstrate that there is a substantial degree of molecular recognition in the processing of the natural diketide chain elongation intermediate. Exogenously added (2*S*,3*R*)-2-methyl-3-hydroxypentanoic acid *N*-acetylcysteamine thioester is exclusively recognized by its cognate β-ketoacyl-acyl carrier protein synthase domain in module 2 (KS2). Labeled diketide specifically acylated DEBS1 in crude protein extracts and limited proteolysis localized the binding to module 2. The precise site of acylation in DEBS1 was established by the finding that a Cys2200Ala mutant of DEBS1, lacking the KS2 active-site cysteine, did not undergo acylation by the diketide. Pretreatment of the wild-type protein with the β-ketoacyl-ACP synthase inhibitor cerulenin also blocked acylation. These results indicate that in addition to the purely organizational consequences resulting from the order of active-site domains, the programming of polyketide biosynthesis by modular PKSs involves a substantial level of molecular recognition. This conclusion has important implications for the use of PKSs to rationally design novel polyketides.

Erythromycin A is a broad-spectrum macrolide antibiotic whose parent polyketide, 6-deoxyerythronolide B (**1**), is synthesized by three multifunctional proteins known collectively as 6-deoxyerythronolide B synthase (DEBS1, DEBS2, and DEBS3;[1] see Figure 1) (Cortes et al., 1990; Donadio et al., 1991; Caffrey et al., 1992). These proteins contain at least 28 catalytic domains where the aglycon skeleton is synthesized from one propionyl primer and six methylmalonyl thioester chain extension units (Cane et al., 1981, 1986). The methylmalonyl thioesters are decarboxylated in condensation reactions leading to β-ketoacylthioester intermediates, which are further reduced in reactions resembling fatty acid synthesis (Hopwood & Sherman, 1990).

Polyketides are diverse in structure and have many biological activities; there is therefore a strong interest in the use of polyketide synthase (PKS) systems to prepare novel, rationally designed products. Structural diversity is achieved in nature by varying the thioester primers and chain extension units used by the PKS and by altering the oxidation level and stereochemistry of intermediates before further condensations. In modular PKSs it appears that the order of functional domains is largely reflected in the sequence of reactions being carried out on the polyketide chain. Thus a major component of the programming of this type of polyketide biosynthesis is essentially organizational. It is tantalizing to speculate that the addition, removal, replace-

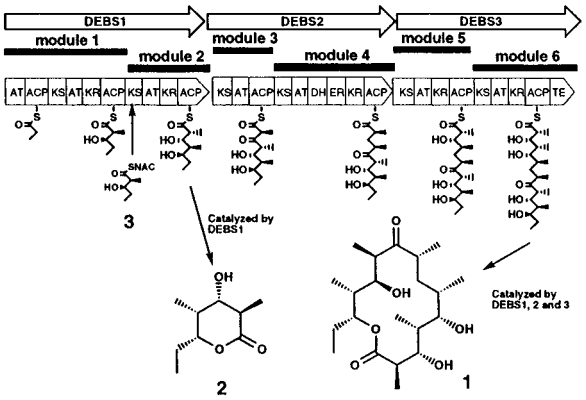

FIGURE 1: Biosynthesis of the polyketide skeleton of erythromycin A. The compound 6-deoxyerythronolide B (**1**) is synthesized by three proteins, DEBS1, -2, and -3. Each protein contains two modules for a round of chain elongation and subsequent reduction. Protein domains are indicated by the following abbreviations: acyl transferase (AT), acyl carrier protein (ACP), β-ketoacyl-ACP synthase (KS), ketoreductase (KR), dehydratase (DH), enoyl reductase (ER), and thioesterase (TE). When DEBS1 is expressed alone, or with a TE domain fused at the C-terminus, synthesis of (2*S*,3*R*,4*S*,5*R*)-2,4-dimethyl-3,5-dihydroxy-*n*-heptanoic acid-δ-lactone (**2**) is observed. (2*S*,3*R*)-2-Methyl-3-hydroxypentanoic acid *N*-acetyl cysteamine thioester (**3**) is converted to the triketide lactone **2** or to **1** by DEBS1+TE or DEBS1+2+3, respectively.

† This research was supported by grants from the National Institutes of Health (CA-66736 to C.K. and GM-22172 to D.E.C.), by a National Science Foundation Young Investigator Award (to C.K.), and by a David and Lucile Packard Fellowship for Science and Engineering (to C.K.).

* Corresponding authors.
‡ Brown University.
§ Stanford University.

⊗ Abstract published in *Advance ACS Abstracts,* October 15, 1996.

[1] Abbreviations: DEBS, 6-deoxyerythronolide B synthase; KS, β-ketoacyl-acyl carrier protein synthase; PKS, polyketide synthase; AT, acyl transferase; TE, thioesterase; ACP, acyl carrier protein; NAC, *N*-acetylcysteamine, KR, ketoreductase.

S0006-2960(96)01972-1 CCC: $12.00

ment, or rearrangement of enzymatic domains will produce new polyketide products. The success of such a strategy depends upon the balance between substrate recognition and permissiveness characteristic of each of the enzymatic activities.

Genetic studies have shown that it is possible to delete or inactivate some functional domains from DEBS and achieve synthesis of unnatural products (Donadio et al., 1991, 1993; Kao et al., 1994, 1995, 1996; Cortes et al., 1995; Bedford et al., 1996). Likewise, the ability of DEBS to use unnatural substrates such as acetyl-CoA and butyryl-CoA as starter units also shows the flexibility of the PKS in processing anomalous substrates and chain-elongation intermediates (Pieper et al., 1995a, 1996). In contrast, however, mutational studies, such as inactivation of the dehydratase of module 4, have yielded no detectable product (Leadlay et al., 1993). Thus, a better understanding of the substrate recognition features within individual domains and modules is paramount in order to rationally guide future efforts in genetically engineered biosynthesis of novel polyketides.

This study has investigated the molecular recognition properties of the two β-ketoacyl-ACP synthase (KS) domains of DEBS1. DEBS1, which contains two modules, carries out two cycles of polyketide chain elongation and processing, resulting in the synthesis of a triketide (Figure 1). In the presence of DEBS2, this linear triketide is transferred to module 3 in DEBS2 for further chain elongation, while DEBS1 alone, or a modified form with a thioesterase domain fused to its C-terminus (DEBS1+TE), synthesizes the corresponding cyclized triketide lactone (**2**) both *in vitro* and *in vivo* (Kao et al., 1995; Pieper et al., 1995b; Cortes et al., 1995; Wiesmann et al., 1995). Here we attempt to identify the domain(s) within DEBS1 that recognize an exogenously added polyketide intermediate. Specifically, we ask whether the two KS domains can discriminate between substrates in preparation for the condensation reaction taking place at each domain. Using radioactive (2*S*,3*R*)-2-methyl-3-hydroxypentanoic acid *N*-acetylcysteamine thioester (**3**), a substrate for the condensation normally catalyzed by KS2 (Cane et al., 1995; Kao et al., 1996), we show that this compound is specifically recognized by KS2, resulting in the acylation of the active-site cysteine.

MATERIALS AND METHODS

Bacterial Strains, Growth Conditions, and Protein Preparation. Three recombinant strains of *Streptomyces coelicolor* CH999 containing plasmids encoding the DEBS1 (pCK9), DEBS1+TE ($KS1^0$, pCK16), and DEBS1+TE ($KS2^0$, pCK17) (Kao et al., 1995, 1996) genes from *Saccharopolyspora erythraea* were used in this study. Bacteria were cultured as described previously (Pieper et al., 1995b). DEBS1 protein was substantially purified from crude cell extracts using a three-step procedure involving ammonium sulfate precipitation, gel filtration, and ion-exchange chromatography based on a modification of the method previously described (Pieper et al., 1996) and used in the experiments below in concentrations of 5−8 μg/μL.

Acylation Studies. [1-^{14}C]Propionyl-CoA (2.0 GBq/mmol) was synthesized as described previously (Patel & Walt, 1987). (2*S*,3*R*)-[1-^{14}C]-2-Methyl-3-hydroxypentanoic acid *N*-acetylcysteamine thioester (diketide-NAC) (681 MBq/mmol) was synthesized essentially as previously described, except that sodium [1-^{14}C]propionate was treated with 1.5 equiv of pivaloyl chloride (THF, 24 h, room temperature) to generate the corresponding mixed anhydride, which was used directly for preparation of [1-^{14}C]propionyl-(4′*S*)-4-benzyl-2-oxazolidinone (Cane & Yang, 1987; Cane et al., 1995). [DL-2-^{14}C]Methylmalonyl-CoA (2.1 GBq/mmol) and [^{3}H]cerulenin (prepared from ^{3}H exchange in $^3H_20$, 7.4 GBq/mmol) were obtained from ARC and Amersham, respectively. Protein extracts (10−20 μL) were added directly to tubes containing [^{14}C]propionyl-CoA (6 μL, 11 kBq) and methylmalonyl-CoA (6 μL, 11 kBq) and typically incubated on ice for 10 min. [^{14}C]Diketide-NAC (25 kBq) and [^{3}H]-cerulenin (37 kBq) were added to tubes, and solvent was evaporated under a stream of nitrogen before addition of protein extracts. Extracts acylated with the diketide-NAC were incubated for 30 min at 30 °C, whereas cerulenin acylations were carried out for 30 min on ice. Experiments were also performed where DEBS1-containing extracts were pretreated with 500 μM unlabeled cerulenin (Sigma). After incubation at 5 °C for 1 h, the protein solution was transferred to a second tube where radioactive diketide-NAC had been previously evaporated to dryness. This tube was then further incubated at 30 °C for 30 min.

All acylation reactions were terminated by the addition of SDS−PAGE sample buffer (4 μL) without reducing agent, and proteins were separated using SDS−4% or 5% PAGE at 80 V at 5 °C. In the case of ^{14}C isotopes, the gels were electroblotted onto PVDF [poly(vinylidene difluoride)] membranes (Immobilon, Millipore) overnight at 150 mA at 5 °C. Membranes were air-dried and exposed to phosphoimager screens (Bio-Rad) for 2 days. Gels containing ^{3}H-labeled proteins were fixed in 2-propanol/acetic acid/H_2O (25:15:60 v/v/v) for 30 min before incubation in Amplify (Amersham) for 30 min. The gels were dried and exposed to X-ray film for 2 days.

Limited proteolytic degradation of DEBS1 (20 μL) was carried out using TPCK-trypsin (0.03 mg/mL, 8.3 units, Pierce) or elastase (0.005 mg, 0.016 unit, Boehringer Mannheim) for various time intervals at 30 °C before incubation with radioactive acylating reagent for 10 min. Again reactions were terminated by the addition of SDS−PAGE sample buffer without reducing agent and proteins separated by SDS−7.5% PAGE gels before transfer to PVDF membranes and phosphoimaging screens.

RESULTS

Diketide-NAC Acylates DEBS1 in Crude Extracts from Recombinant S. coelicolor CH999/pCK9. The specificity of diketide acylation of DEBS1 was first examined in preliminary experiments using desalted extracts of DEBS1 after the ammonium sulfate precipitation step. Control experiments established that this crude extract, which exhibited a major band on SDS−PAGE at ca. 350−370 kDa, was able to synthesize 2,4-dimethyl-3,5-dihydroxy-*n*-heptanoic δ-lactone (**2**) when provided with the substrates methylmalonyl-CoA, propionyl-CoA, and NADPH, consistent with the protein extract containing active DEBS1 (Pieper et al., 1995b). When these extracts were incubated at 4 °C for 5- and 30-min intervals with the radioactively labeled propionyl-CoA, DEBS1 underwent acylation, as expected (Pieper et al., 1995a). Using [^{14}C]diketide-NAC (**3**) and incubation temperatures of 30 °C, acylation of DEBS1 was also observed.

Diketide-NAC Specifically Binds to Module 2 in DEBS1. Limited proteolysis of gel filtration-purified extracts of

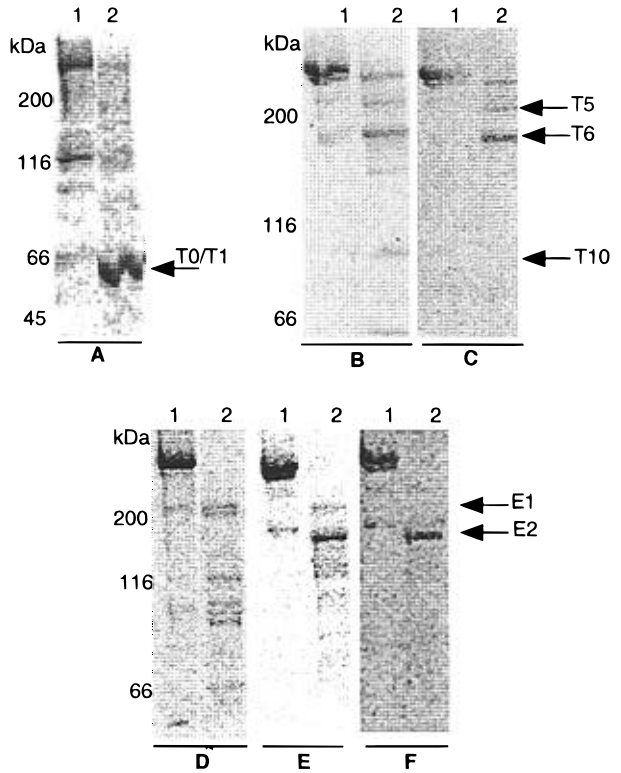

FIGURE 2: Acylation of fragments derived from limited proteolysis of DEBS1. After digestion with either trypsin (panels A−C) or elastase (panels D and F) for 30 min, fragments were acylated using propionyl-CoA (panels A and D, lane 2), methylmalonyl-CoA (panel B, lane 2) or diketide-NAC **3** (panels C and F, lane 2). Panel E, lane 2, shows treatment with elastase for 15 min prior to acylation with methylmalonyl-CoA. Lane 1 in each panel shows acylation of undigested protein. Fragment nomenclature is according to Aparicio et al. (1994). Important fragments include T0 and T1 which contain the propionyl-CoA loading domain (AT0-ACP0), and T5 (KR1-ACP1-KS2-AT2-KR2-ACP2) and T6 (ACP1-KS2-AT2-KR2-ACP2), both of which are major fragments labeled by methylmalonyl-CoA and diketide-NAC. Important elastase fragments include E1, containing both a methylmalonyl-CoA and propionyl-CoA binding domain (AT0-ACP0-KS1-AT1-KR1-ACP1), and E2, corresponding to KS2-AT2-KR2-ACP2, the fragment acylated by both methylmalonyl-CoA and diketide-NAC **3**.

DEBS1 was carried out to determine the position(s) of acylation by the diketide-NAC. Using known trypsin and elastase peptide maps of DEBS1 (Aparicio et al., 1994), the fragments containing diketide binding domains were readily identified. Thus TPCK-trypsin was used to partially digest DEBS1 for time periods ranging from 15 to 60 min. (Data for 30-min digestions are shown in Figure 2.) After each digestion, acylations were carried out for 10 min at either 5 °C for propionyl-CoA or methylmalonyl-CoA or at 30 °C for the diketide-NAC **3**. Consistent with previous studies (Aparicio et al., 1994; Marsden et al., 1994), in a control experiment propionyl-CoA acylated a tryptic protein fragment of mass ca. 50−60 kDa (Figure 2, panel A, fragment T0/T1) that has previously been shown to contain the N-terminal acyl transferase and ACP loading domains of module 1 (AT0-ACP0) (Aparicio et al., 1994).

A second control experiment using methylmalonyl-CoA showed a more complicated labeling pattern due to the rapid degradation of labeled protein fragments as well as hydrolysis of the attached methylmalonyl ester, essentially the same pattern previously reported for this substrate (Aparicio et al., 1994; Marsden et al., 1994). The major labeled tryptic peptides were a fragment, ca. 220 kDa, that decreased in intensity with time (Figure 2, panel B, fragment T5) and a second peptide (170 kDa, fragment T6) that increased in intensity over time. From the known peptide map of DEBS1 (Aparicio et al., 1994), the larger of the two labeled fragments has been assigned to KR1-ACP1-KS2-AT2-KR2-ACP2, whereas the 170-kDa protein contained ACP1-KS2-AT2-KR2-ACP2 domains. An additional faint band of ca. 95 kDa (T10, AT0-KS1), corresponding to acylation in module 1, was also visible (Figure 2, panel B, lane 2).

Incubation of [1-^{14}C]-(2*S*,3*R*)-2-methyl-3-hydroxypentanoic acid *N*-acetylcysteamine thioester (**3**) with partially trypsinized DEBS1 gave two labeled peptide fragments of molecular mass 220 kDa (T5, KR1-ACP1-KS2-AT2-KR2-ACP2) and 170 kDa (T6, ACP1-KS2-AT2-KR2-ACP2), suggesting exclusive acylation within module 2 (Figure 2, panel C). Analogous to the methylmalonyl-CoA acylation experiment, the 220-kDa fragment decreased in intensity over time, and after 60 min of trypsin digestion, this fragment was no longer visible (data not shown). No peptide fragments containing only module 1 domains were acylated by the labeled diketide, in contrast with the results of the methylmalonyl-CoA and propionyl-CoA control experiments.

Further refinement in the localization of the diketide binding site was achieved by elastase peptide mapping. Again, control acylation experiments using methylmalonyl-CoA and propionyl-CoA were carried out to identify generated peptide fragments. Proteolytic degradation of DEBS1 with elastase has been shown to cleave the protein between the junction of modules 1 and 2 (Aparicio et al., 1994). This is evident by the appearance of two major fragments of 210 kDa (fragment E1) and 160 kDa (fragment E2) that both can be acylated by methylmalonyl-CoA (Figure 2, panel E) (Marsden et al., 1994). The 210-kDa fragment is subject to further degradation whereas the 160-kDa fragment is stable in the presence of elastase under the experimental conditions used (Aparicio et al., 1994). Propionyl-CoA acylated the 210-kDa fragment, corresponding to the loading domain of module 1, and not the 160-kDa module 2 fragment (Figure 2, panel D). The sizes of all these acylated fragments were also compared with the sizes of fragments generated by elastase degradation of the purified DEBS1 protein (results not shown).

Labeled diketide-NAC **3** exclusively acylated the 160-kDa elastase fragment of DEBS1, as can be seen in Figure 2, panel F. This labeled fragment (KS2-AT2-KR2-ACP2) did not decrease in intensity during the digestion time, indicating again that it was stable to elastase under the conditions used in the experiment, consistent with previous reports (Aparicio et al., 1994).

Diketide-NAC Does Not Bind to a β-Ketoacyl-ACP Synthase 2 (KS2^0) Mutant of DEBS1. The expected site of binding of diketide-NAC in the KS2-AT2-KR2-ACP2 fragment was the active-site cysteine of KS2. To test this hypothesis further, we used two mutants of DEBS1+TE, modified by site-directed mutagenesis in KS1 and KS2, respectively (Kao et al., 1996), to study the binding of the diketide-NAC to DEBS1. In these mutant proteins the active-site cysteine of either KS1 (C729) or KS2 (C2200) has been changed to an alanine residue, rendering each enzyme inactive for triketide lactone synthesis from propionyl-CoA and methylmalonyl-CoA (Kao et al., 1996). Triketide lactone formation can in fact be restored by incubation of the KS1^0 protein with diketide-NAC (**3**) in the presence of methylmalonyl-CoA and NADPH, indicating that module 2

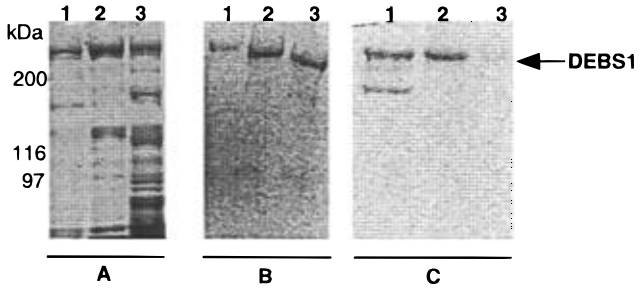

FIGURE 3: Acylation of KS mutants of DEBS1. Wild-type DEBS (lane 1), $KS1^0$ (lane 2), and $KS2^0$ (lane 3) protein extracts were acylated by propionyl-CoA (panel B) and diketide-NAC (panel C), separated by SDS−4% PAGE, and blotted to PVDF membranes. Acylations were carried out using 10 μL of protein extracts. Panel A shows the Coomassie blue-stained gel of the protein extracts (5 μL) used in the experiment.

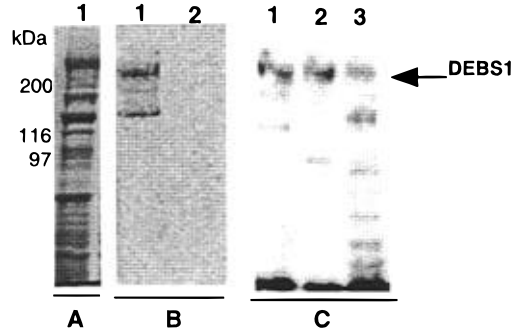

FIGURE 4: Effect of cerulenin on acylation of DEBS1 by diketide. Protein extracts pretreated with 500 μM cerulenin showed no binding of radioactive diketide (panel B, lane 2). Acylation of untreated DEBS1 by diketide-NAC is shown in panel B, lane 1, as a control. Commassie blue-stained gel of the DEBS1 protein preparation used in the experiment is shown in panel A. [^{3}H]-Cerulenin bound to DEBS1, $KS1^0$ and $KS2^0$, as observed by fluorography (panel C, lanes 1, 2, and 3).

in this mutant remains fully functional (Kao et al., 1996). Furthermore, a heterodimer of the $KS1^0$ and $KS2^0$ proteins also supports triketide lactone formation from propionyl-CoA, methylmalonyl-CoA, and NADPH (Kao et al., 1996).

Due to the lower expression of the $KS1^0$ and $KS2^0$ mutants, all proteins were further purified by ion-exchange chromatography before parallel acylation experiments were carried out. Propionyl-CoA acylation of both wild-type and mutant proteins was used as a control to verify the integrity of the proteins used in this experiment. As seen in Figure 3, panel B, all three protein preparations were acylated by [^{14}C]propionyl-CoA. In contrast, only the wild-type protein and the $KS1^0$ mutant, which lacks the active-site cysteine of KS1, were acylated by the diketide (Figure 3, panel C). Even when twice as much protein was treated with [^{14}C]diketide **3**, no diketide binding to DEBS1+TE ($KS2^0$) was observed (data not shown). This result is consistent with specific covalent binding of diketide **3** to the active-site cysteine of KS2, the same site that normally would be acylated by endogenously generated diketide in preparation for the second condensation reaction carried out by DEBS1.

A second, lower molecular weight protein band in these protein preparations also appeared to be acylated by labeled diketide. It is likely that the latter is a proteolytic degradation product of DEBS1. Evidence for this conclusion includes (i) the complete lack of labeling of the corresponding peptide fragment in the $KS2^0$ protein preparations and cerulenin competition experiments (see below) and (ii) the increased size of the corresponding fragment in the $KS1^0$ mutant extracts (data not shown) compared with the wild-type protein preparation. Since the mutant proteins include the thioesterase domain from DEBS3, the latter proteins have a slightly higher mass compared with the wild-type DEBS1 protein. If proteolytic cleavage ocurs on the N-terminal side of the diketide binding site, such a fragment of higher mass would be derived from the acylated DEBS1+TE ($KS1^0$) protein. Consistent with this notion is the observed lack of propionyl-CoA acylation of this proteolysis product, which would lack the N-terminal AT loading domain (Figure 3, panel B).

Cerulenin Binds to DEBS1. The formation of the triketide lactone by DEBS1+TE is inhibited by the fatty acid analog cerulenin (Pieper et al., 1995b). This irreversible inhibitor has been shown to acylate the active-site cysteine of the ketosynthases of fatty acid synthesis (Kauppinen et al., 1988), proteins homologous to the KS domains of polyketide synthases. If the diketide were binding at an active-site cysteine, pretreatment of wild-type protein with cerulenin would be expected to prevent diketide binding to the enzyme.

As shown in Figure 4, panel B, lane 2, pretreatment of DEBS1 with 500 μM cerulenin for 1 h on ice completely inhibited acylation of the protein by [^{14}C]diketide-NAC. This is consistent with binding of the diketide to a KS active-site cysteine that is blocked against acylation by irreversible attachment of cerulenin. As expected, [^{3}H]cerulenin alkylated DEBS1, DEBS1+TE ($KS1^0$), and DEBS1+TE ($KS2^0$), as is evident in the fluorogram shown in Figure 4, panel C. Additional proteins were also labeled by cerulenin in these crude preparations, although higher specificity for DEBS1 for cerulenin binding was observed when the protein was incubated in the presence of 500 μM cerulenin at 5 °C rather than at 30 °C (data not shown).

DISCUSSION

The specific acylation of the active-site cysteine of the β-ketoacyl-ACP synthase domain (KS2) of module 2 by (2*S*,3*R*)-[1-^{14}C]-2-methyl-3-hydroxypentanoic acid *N*-acetylcysteamine thiester, a thioester analog of its natural substrate, is evidence for seletive molecular recognition of polyketide chain elongation intermediates within a modular polyketide synthase. Previous acylation studies have shown that the acyltransferase loading domains (AT0) of DEBS1 can tolerate a variety of different primers, including acetate and butyrate, in place of the natural propionyl-CoA substrate (Pieper et al., 1995a; Marsden et al., 1994; Aparicio et al., 1994). By contrast, the AT domains responsible for loading the methylmalonyl chain extension units appear to have a strict preference for (2*S*)-methylmalonyl-CoA (Marsden et al., 1994). Little was known, however, about the molecular recognition of polyketide intermediates during polyketide chain elongation.

Previous work by our group has demonstrated the intact incorporation of exogenously added (2*S*,3*R*)-2-methyl-3-hydroxypentanoic acid *N*-acetylcysteamine thioester (**3**) into 6-deoxyerythronolide B and the triketide lactone (**2**), in both *in vivo* and *in vitro* experiments, respectively, with no abnormal polyketides being detected (Pieper et al., 1995a; Cane et al., 1995). These results clearly suggested the ability of DEBS1 to recognize and properly process the analog of its normal diketide chain elongation intermediate. The domain where this discrimination resided was not known, however, nor was it known whether covalent attachment of

the exogenously added NAC-thioester to the KS2 active site was a prerequisite for generation of the triketide product. Moreover, the possibility still existed that KS1 might be acylated by the diketide in a competing, nonproductive side reaction. This study has clearly established that selection of the natural substrate for condensation is mediated by the cognate KS domain. This result is fully consistent with incubations of $KS1^0$ and $KS2^0$ with diketide-NAC, methylmalonyl-CoA, and NADPH, which showed that the $KS1^0$ mutant protein could synthesize the triketide lactone **2** from the diketide intermediate whereas the $KS2^0$ mutant remained completely inactive (Kao et al., 1996).

Under normal catalytic conditions, the (2*S*,3*R*)-2-methyl-3-hydroxypentanoyl thioester is initially generated attached to the ACP domain of module 1 (ACP1), from whence it is transferred to the active site cysteine of the module 2 ketosynthase (KS2) prior to the next round of polyketide chain elongation. Peptide mapping using elastase showed that exogenously added diketide-NAC thioester did not acylate the ACP1 domain. Acylation of intact DEBS1 followed by partial proteolysis gave compatible results (data not shown). Since proteolytic digestion of DEBS1 with elastase prior to incubation with [^{14}C]diketide-NAC resulted in exclusive acylation of the peptide fragment corresponding to module 2, it is evident that the exogenously added diketide analog can directly acylate the KS2 domain, without a requirement for prior attachment to the ACP1 domain. *N*-Acetylcysteamine thioesters have been previously used as surrogate ACP analogs for *in vitro* fatty acid biosynthetic reactions (Kass, 1969). In spite of the fact that the reduced diketide chain elongation intermediate is normally transferred directly from ACP1 to the KS2 domain, it can now be concluded that an additional level of molecular recognition is clearly operative during this process. Similar discrimination presumably plays a role in analogous transthioesterifications between downstream ACP domains and the KS domains of the next biosynthetic module, both within the same and between different DEBS subunits.

These results have important implications for the use of modular polyketide systems to engineer new and novel polyketide products. The extent to which modules can be rearranged, removed, or added will depend on the permissiveness of the individual domains that must process unnatural substrates and derived intermediates. Some catalytic domains, such as the AT0 loading domain, are relatively nonselective, while other AT domains appear to have a strict specificity. Thus the AT domains of DEBS will process only the 2*S* enantiomer of methylmalonyl-CoA (Marsden et al., 1994), and individual AT domains of the rapamycin synthase control the choice of malonyl- or methylmalonyl chain extension units (Haydock et al., 1995).

The current study has shown that there is a substantial degree of molecular recognition in the processing of the natural diketide chain elongation intermediate. Exogenously added (2*S*,3*R*)-2-methyl-3-hydroxypentanoyl-NAC thioester is exclusively recognized by its cognate KS2 active site and does not acylate the homologous KS1 active-site Cys, although sequence comparisons show that KS1 and KS2 domains are 61% identical and 77% similar at the amino acid level. In spite of the ability of the KS2 domain to recognize its natural substrate, there must be considerable flexibility in substrate specificity, since this same domain will process unnatural diketides of varying chain length and oxidation level, either generated *in situ* by module 1 or added exogenously (Pieper et al., 1995a, 1996; Luo et al., 1996). Studies on the structural basis and the intrinsic limits of this discrimination are in progress.

As more information on the PKS protein structure and the degree of substrate flexibility becomes available, the more likely it will be that rational design choices to make unnatural polyketides will be successful. It is already becoming clearer that the type I modular PKS systems have a higher level of control other than the purely organizational consequences resulting from the ordering of individual catalytic domains. This may be circumvented by the shuffling of domains and modules between PKS systems to produce hybrid proteins able to make unusual polyketides.

ACKNOWLEDGMENT

Penny von Wettstein-Knowles is thanked for the gift of the [^{3}H]cerulenin.

REFERENCES

Aparicio, J. F., Caffrey, P., Marsden, A. F. A., Staunton, J., & Leadlay, P. F. (1994) *J. Biol. Chem. 269*, 8524−8528.
Bedford, D., Jacobsen, J. R., Luo, G., Cane, D. E., & Khosla, C. (1996) *Chem. Biol. 3*, 827−831.
Caffrey, P., Bevitt, D. J., Staunton, J., & Leadlay, P. F. (1992) *FEBS Lett. 304*, 225−228.
Cane, D. E., & Yang, C. (1987) *J. Am. Chem. Soc. 109*, 1255−1257.
Cane, D. E., Hasler, H., & Liang, T.-C. (1981) *J. Am. Chem. Soc. 103*, 5960−5962.
Cane, D. E., Liang, T. C., Taylor, P. B., Chang, C., & Yang, C. C. (1986) *J. Am. Chem. Soc. 108*, 4957−4964.
Cane, D. E., Luo, G., Khosla, C., Kao, C. M., & Katz, L. (1995), *J. Antibiot. 48*, 647−651.
Cortes, J., Haydock, S. F., Roberts, G. A., Bevitt, D. J., & Leadlay, P. F. (1990) *Nature 348*, 176−178.
Cortes, J., Weismann, K. E. H., Roberts, G. A., Brown, M. J. B., Staunton, J., & Leadlay, P. F. (1995) *Science 268*, 1487−1489.
Donadio, S., Staver, M. J., McAlpine, J. B., Swanson, S. J., & Katz, L. (1991) *Science* 252, 675−679.
Donadio, S., McAlpine, J. B., Sheldon, P. J., Jackson, M., & Katz, L. (1993) *Proc. Natl. Acad. Sci. U.S.A. 90*, 7119−7123.
Haydock, S. F., Aparicio, J. F., Molnar, I., Schwecke, T., Khaw, L. E., König, A., Marsden, A. F. A., Galloway, I. S., Staunton, J., & Leadlay, P. F. (1995) *FEBS Lett. 374*, 246−248.
Hopwood, D. A., & Sherman, D. H. (1990) *Annu. Rev. Genet. 24*, 37−66.
Kao, C. M., Luo, G., Katz, L., Cane, D. E., & Khosla, C. (1994) *J. Am. Chem. Soc. 116*, 11612−11613.
Kao, C. M., Luo, G., Katz, L., Cane, D. E., & Khosla, C. (1995) *J. Am. Chem. Soc. 117*, 9105−9106.
Kao, C. M., Pieper, R., Cane, D. E., & Khosla, C. (1996) *Biochemistry 35*, 12363−12368.
Kass, L. R. (1969) In *Methods in Enzymology* (Lowenstein, J. M., Ed.) Vol. XIV, pp 73−88, Academic Press, New York.
Kauppinen, S., Siggaard-Andersen, M., & von Wettstein-Knowles, P. (1988) *Carlsberg Res. Commun. 53*, 357−370.
Leadlay, P. F., Staunton, J., Aparicio, J. F., Bevitt, D. J., Caffrey, P., Cortes, J., Marsden, A., & Roberts, G. A. (1993) *Biochem. Soc. Trans. 21*, 218−222.
Luo, G., Pieper, R., Rosa, A., Khosla, C., & Cane, D. E. (1996) *Bioorg. Med. Chem. 4*, 995−999.
Marsden, A. F. A., Caffrey, P., Aparicio, J. F., Loughran, M. S., Staunton, J., & Leadlay, P. F. (1994) *Science 263*, 378−380.
Patel, S. S., & Walt, D. R. (1987) *J. Biol. Chem. 262*, 7132−7134.
Pieper, R., Luo, G., Cane, D. E., & Khosla, C. (1995a) *J. Am. Chem. Soc. 117*, 11373−11374.
Pieper, R., Luo, G., Cane, D. E., & Khosla, C. (1995b) *Nature 378*, 263−266.
Pieper, R., Ebert-Khosla, S., Cane, D., & Khosla, C. (1996) *Biochemistry 35*, 2054−2060.
Wiesmann, K. E. H., Cortes, J., Brown, M. J. B., Cutter, A. L., Staunton, J., & Leadlay, P. F. (1995) *Chem. Biol. 2*, 583−589.

BI961972F