Analysis of Covalently Bound Polyketide Intermediates on 6-Deoxyerythronolide B Synthase by Tandem Proteolysis−Mass Spectrometry†

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ABSTRACT: Polyketide natural products are biosynthesized via successive chain-elongation events mediated by elaborate protein assemblies. Facile detection of protein-bound intermediates in these systems will increase our understanding of enzyme reactivity and selectivity. We have developed a tandem proteolysis/mass spectrometric method for monitoring substrate loading and elongation in 6-deoxyerythronolide B synthase (DEBS), responsible for production of the macrolide precursor to erythromycin. Information regarding ketosynthase loading and polyketide unit elongation is readily acquired without need for complex protein or small molecule labels. A panel of structurally related substrates is evaluated through competition experiments and kinetic assays using LC-MS to resolve closely related species. Strong stereochemical effects are observed for ketosynthase substrate specificity. Semiquantitative kinetic analyses allow the resolution of the effects of structural and stereochemical changes on the individual ketosynthase-catalyzed steps of acyl-enzyme formation and polyketide chain extension.

Nature exhibits an impressive ability to manufacture a wide range of structurally unique small molecules through selective and efficient biochemical processes. Mechanistic interrogation of biosynthetic steps has resulted in a variety of important discoveries ranging from development of new bioinspired reactions to genetic reprogramming of these pathways toward generation of novel natural product analogues (*1*−*6*). The practical realization of combinatorial biosynthetic techniques could benefit from in-depth understanding of substrate specificity and enzyme reactivity. Direct investigations of basic structural and functional requirements within intricate biosynthetic systems are more and more essential as the questions posed become increasingly complex.

Polyketides are a diverse and pharmaceutically important class of complex natural products constructed through sequential condensation reactions involving multienzyme assemblies termed polyketide synthases (PKSs)[1] (*7*, *8*). Modular polyketide synthases consist of multiple, covalently tethered catalytic domains organized in large polypeptides which specifically associate to generate a polymerization scaffold mechanistically reminiscent of an industrial assembly line. The machinery responsible for production of 6-deoxyerythronolide B, the macrolide precursor to erythromycin, has been extensively studied over the past 2 decades, providing much of our current understanding of these systems (*9*).

Despite the obvious structural diversity among polyketide products of modular PKSs, their biosynthesis occurs through a highly conserved, basic set of biochemical transformations. Each PKS subunit consists of an individual module, constructed from a combination of catalytic domains, responsible for a single round of polyketide chain elongation and functional group modification. The first step is the formation of an acyl-enzyme intermediate between the growing polyketide substrate and the active site cysteine of the ketosynthase (KS) domain (Figure 1). A malonyl or methylmalonyl extender unit, attached to the acyl carrier protein (ACP) domain of the same module under control of a highly specific acyl transferase (AT) domain, is then coupled to the KS-bound polyketide chain by a KS-catalyzed decarboxylative condensation reaction. The resulting β-ketoacylthioester may then undergo a series of transformations while still anchored to the ACP, with the ultimate level of oxidation determined by the precise combination of ketoreductase (KR), dehydratase (DH), and enoyl reductase (ER) domains present in the module between the AT and ACP domains. Each such modification reaction is stereospecific, with the potential for dictating up to two stereocenters in the final polyketide product. Once fully processed, the newly elongated polyketide intermediate is transferred from the ACP to the KS domain of the downstream module via transthioesterification. The polyketide chain elongation process is repeated through the final module where cleavage from the protein takes place either through hydrolysis or by thioesterase- (TE-) mediated macrolactonization.

Recent efforts in our laboratory have resulted in successful engineering of a catalytically active [KS][AT] didomain

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[1] Abbreviations: PKS, polyketide synthase; DEBS, 6-deoxyerythronolide B synthase; KS, ketosynthase; AT, acyl transferase; KR, β-ketoreductase; DH, dehydratase; ER, enoyl reductase; ACP, acyl carrier protein; TE, thioesterase; SNAC, *N*-acetylcysteamine; LC-MS, liquid chromatography−mass spectrometry.

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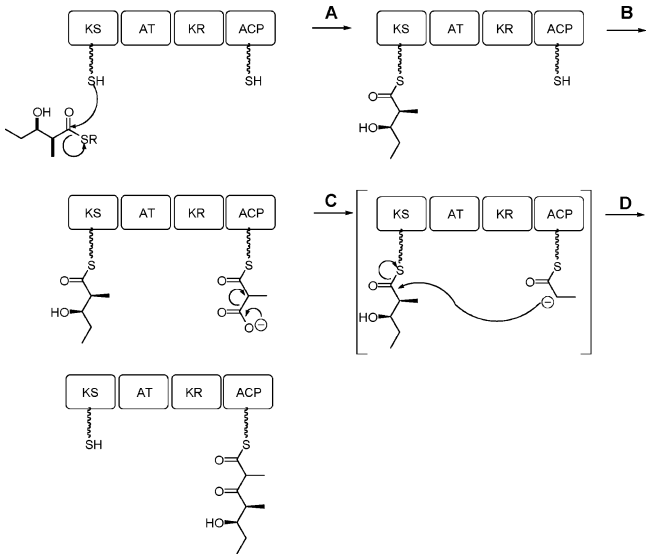

FIGURE 1: Schematic diagram of polyketide assembly. Steps: (A) Transthioesterification loads diketide unit on ketosynthase domain. (B) Acyl carrier protein is loaded with methylmalonyl extender unit from acyl transferase domain. (C, D) Acyl donor and acceptor units undergo decarboxylative condensations to produce triketide bound to acyl carrier protein. Abbreviations: KS = ketosynthase, AT = acyl transferase, KR = ketoreductase, and ACP = acyl carrier protein.

derived from module 3 of 6-deoxyerythronolide B synthase (DEBS) (*10*). We were initially interested in developing a method to monitor both acyl intermediate loading and elongation for a variety of model substrates. Kelleher and co-workers have utilized various degrees of proteolysis to excise peptidyl carrier protein (PCP) and ACP fragments from intact nonribosomal peptide synthetase (NRPS) and PKS modules. FTMS analysis provided direct evidence for covalently attached acyl-enzyme and elongation products (*11*−*14*). We hypothesized that an analogous method could be used to visualize enzyme-bound polyketides on both the DEBS KS and ACP domains. The ability to resolve small changes in molecular mass allows for simultaneous testing of very similar compounds, thereby allowing facile examination of the substrate specificity of the KS domain. A recent report by Leadlay and co-workers has demonstrated the feasibility of identifying acylated substrates attached to the loading didomain and the first ketosynthase domain of DEBS1 (*15*). This report describes our initial efforts toward direct visualization of the self-acylation and polyketide elongation reactions catalyzed by DEBS module 3.

EXPERIMENTAL PROCEDURES

Materials. Diketide and triketide substrates were prepared by established methods (*16*−*18*). Trypsin and methylmalonyl-CoA were obtained from Sigma-Aldrich (Milwaukee, WI). Ni-NTA affinity resin was purchased from Qiagen (Valencia, CA). HiTrap-Q anion-exchange columns were from Amersham Pharmacia Biotech (Piscataway, NJ). DL-[2-*methyl*-^{14}C]Methylmalonyl-CoA was from American Radiolabeled Chemicals. Thin-layer chromatography (TLC) plates (IB2) were from J. T. Baker.

Expression of [KS][AT] and Holo-ACP from DEBS Module 3. The construction of genes encoding [KS][AT] (pAYC2) and ACP (pVYA05) have been previously described (*10*). Plasmid AYC2 was transformed into *Escherichia coli* BL21(DE3) while plasmid VYA05 was transformed into *E. coli* BAP1. The *E. coli* BAP1 cell line contains a plasmid encoding the Sfp phosphopantetheinyl transferase and was used to effect pantetheinylation of ACP. Cells were grown at 37 °C in LB medium with 100 μg/mL carbenicillin ([KS][AT]) or 50 μg/mL kanamycin (ACP3) to an OD_{600} = 0.6, at which point they were cooled to 18 °C and induced with 0.2−0.5 mM IPTG for 24 h. The cells were harvested by centrifugation (4500*g*, 15 min) and resuspended in lysis/wash buffer (75 mM phosphate, pH 7.6, 300 mM NaCl, 10 mM imidazole). Cells were lysed with sonication (5 × 1 min), and cellular debris was removed by centrifugation (17000*g*, 45 min). Nickel-NTA agarose resin was added directly to the supernatant (8 mL of resin/L of culture) and mixed for 3 h at 4 °C. This resin was poured into a fritted column, washed with 10 column volumes of lysis/wash buffer, and eluted with 3 column volumes of elution buffer (150 mM phosphate, pH 7.6, 50 mM NaCl, 150 mM imidazole). The eluted protein was applied to a HiTrap-Q anion-exchange column and eluted with an increasing linear NaCl gradient. [KS][AT] eluted at approximately 370 mM NaCl, while ACP was recovered in the flow-through. A typical protein yield was 20 mg of [KS][AT]/L of culture volume and 10 mg of ACP/L of culture volume.

Acylation of the Ketosynthase Domain. To a 12.5 μM (50 μL total volume) solution of purified [KS][AT] protein in phosphate buffer (100 mM sodium phosphate, pH = 7.1) was added the appropriate diketide and/or triketide(s) to a final substrate concentration of 5 mM (10 mM for competition experiments). Substrates used in competition experiments were mixed prior to exposure to [KS][AT]. Reactions were allowed to proceed for 1 h at room temperature before proteolysis (see below), unless specified otherwise.

Elongation of KS-Bound Intermediates. To a mixture of [KS][AT] (12.5 μM) in phosphate buffer (as above, 50 μL total volume) was added the appropriate substrate (5 mM total concentration) and tris(2-carboxyethyl)phosphine (TCEP) to a final concentration of 2.5 mM. After incubation for 1 h at room temperature to allow acylation of the KS to go to completion, methylmalonyl-CoA (1 mM final concentration) and *holo*-ACP (50 μM final concentration) were then added and reacted at room temperature for 5, 15, or 30 min followed by trypsin proteolysis (see below). Peak heights, corresponding to active site-containing ACP fragments, were taken directly from mass spectra. Turnover numbers were determined by calculating the percentage of ACP loaded with elongated substrate and multiplying by four, the maximum possible turnover number.

Trypsin Proteolysis of [KS][AT] and Holo-ACP. To each reaction mixture was added trypsin (100 μM solution of ddH_2O) such that the final protease:[KS][AT] molar ratio reached 1:5. In experiments where ACP was present, the final trypsin:total protein molar ratio was 1:12.5. The solution was incubated at 30 °C for 1 h followed by addition of an equal volume of 10% formic acid to quench the reaction. Samples were stored at 4 °C until LC-MS analysis.

HPLC-MS Analysis. Separations were performed on a Surveyor HPLC system (ThermoFinnigan, San Jose, CA). Solvent A was H_2O with 0.1% formic acid; solvent B was acetonitrile with 0.1% formic acid. Samples were chromatographed on a Vydac reverse-phase C-18 polymer column at a flow rate of 200 μL/min, using a gradient of 5−95%

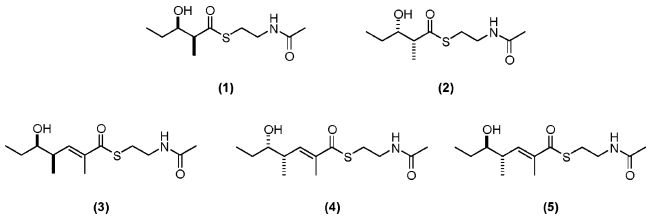

FIGURE 2: Structures of di- and triketides used in loading and elongation assays. Each is used as the *N*-acetylcysteamine thioester, known to be an appropriate surrogate for donor acyl-ACPs, for loading of ketosynthase.

solvent B over 60 min. Mass spectra were collected on an LCQ quadrupole ion trap (ThermoFinnigan, San Jose, CA) mass spectrometer equipped with an electrospray ion source operating in the positive ion mode. The sheath gas was set to 60 (arbitrary units), spray voltage to 4.5 kV, and capillary temperature to 200 °C. Data were processed using Qual-Browser software (Finnigan Inc.).

Radioactive Triketide Lactone Formation. To a mixture of [KS][AT] (12.5 μM) in phosphate buffer (as above, 10 μL total volume at each time point) was added diketide **1** (5 mM final concentration) and tris(2-carboxyethyl)phosphine (TCEP) (2.5 mM final concentration). The solution was incubated for 1 h at room temperature to acylate the KS to completion. DL-[2-*methyl*-^{14}C]Methylmalonyl-CoA (500 μM final concentration) and *holo*-ACP (50 μM final concentration) were then added and allowed to react at room temperature for 5, 15, or 30 min. At each time point the reaction was quenched by adding 20 μL of 0.5 M potassium hydroxide and heating the mixture for 20 min at 65 °C. Hydrochloric acid (10 μL of 1.5 M) was then added, and the mixture was dried in a Speed-vac for 1 h. The pellet was resuspended in 10 μL of ethyl acetate and spotted onto a TLC plate. A 60:40 mixture of ethyl acetate/hexane was used for radioactive TLC, then visualized, and quantified using a Packard phosphorimager. Turnover numbers were determined from the radioactivity of the triketide products.

RESULTS

Substrate Loading of the Ketosynthase Domain. The considerable size of the [KS][AT] fragment from module 3 (ca. 100 kDa) precluded direct detection of covalently bound substrates by mass spectrometry. A method was therefore developed to achieve complete proteolysis of the didomain under conditions expected to minimize thioester hydrolysis. Site-directed mutagenesis has previously been used to identify the active site cysteines of both KS domains of DEBS1, allowing ready prediction of the appropriate proteolytic fragment harboring the corresponding active site Cys of the KS domain of module 3 (*19*, *20*). Treatment of [KS]-[AT] with trypsin (5:1 protein:enzyme molar ratio) at 30 °C for 1 h produced the active site cysteine-containing peptide, ISYTMGLEGPSISVDTA**C**SSSLVALHLAVESLR (MW = 3406), which was unambiguously identified by LC-MS analysis (see Supporting Information). The ability of DEBS module 3 to efficiently process the *N*-acetylcysteamine (SNAC) thioester of (2*S*,3*R*)-2-methyl-3-hydroxypentanoic acid (**1**, Figure 2) is well documented. We therefore initially focused our efforts on acylation of the KS active site with diketide **1**. Incubation of the [KS][AT] didomain with a large excess of substrate for 1 h at ambient temperature resulted

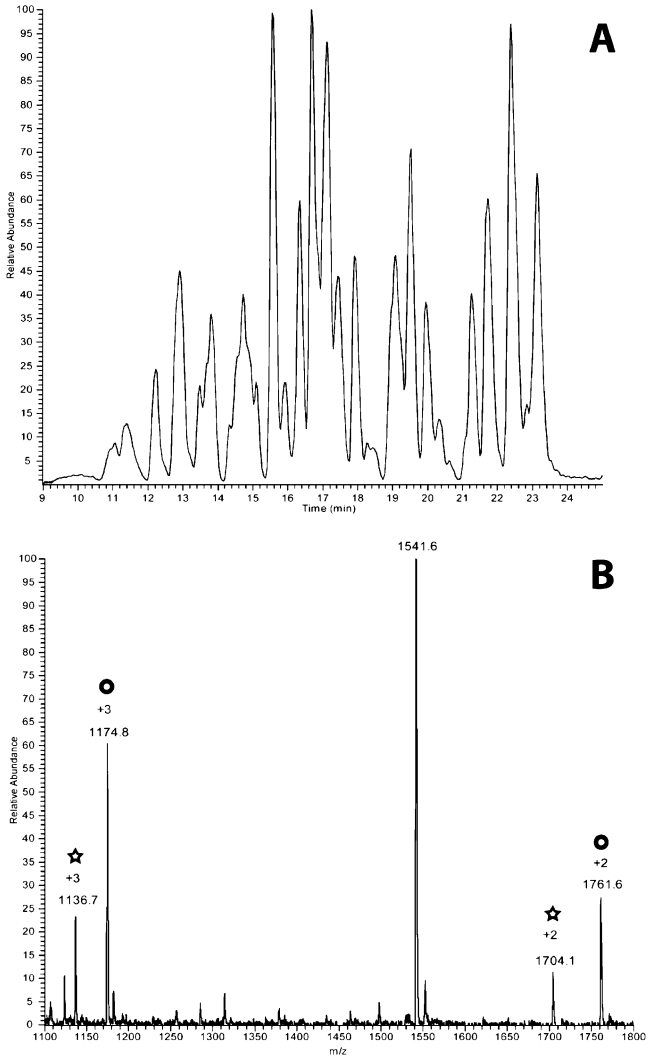

FIGURE 3: KS loading with compound **1**. (A) Total ion count (TIC) profile from LC analysis of [KS][AT] proteolysis with trypsin. (B) Electrospray ionization mass spectrum from (A) (22−24 min) of proteolysis fragments corresponding to unloaded KS (stars) and KS loaded with **1** (circles). Peaks corresponding to $z = +2$ and $+3$ are labeled.

in >75% acylation, as determined by LC-MS (Figure 3). Importantly, the newly formed acylthioester linkage appeared to be stable toward cleavage during both proteolysis and mass spectrometric analysis.

With a sensitive and reliable method in hand to assay KS acylation, we examined the substrate specificity of the ketosynthase domain using pairwise combinations of diketide **1** and individual diastereomeric di- and triketides−SNAC thioesters. Each mixture was incubated with the [KS][AT] didomain for 1 h. Since acylation of the KS domain is irreversible under the conditions used (data not shown), the specificity for each substrate, as measured by the relative proportions of each acyl-enzyme species, was determined from the LC-MS data, as described above (Figure 4). Diketide **1** emerged as the best substrate for the acylation reaction. Interestingly, unsaturated triketide substrates **4** and **5** acylated the KS more rapidly than did triketide **3**. Since **1** and **2** have identical molecular mass, precluding direct competition experiments, the relative specificity for **2** was determined by competition with triketide **3**. As expected, *syn*-diketide **2**, the enantiomer of **1**, was the slowest to react.

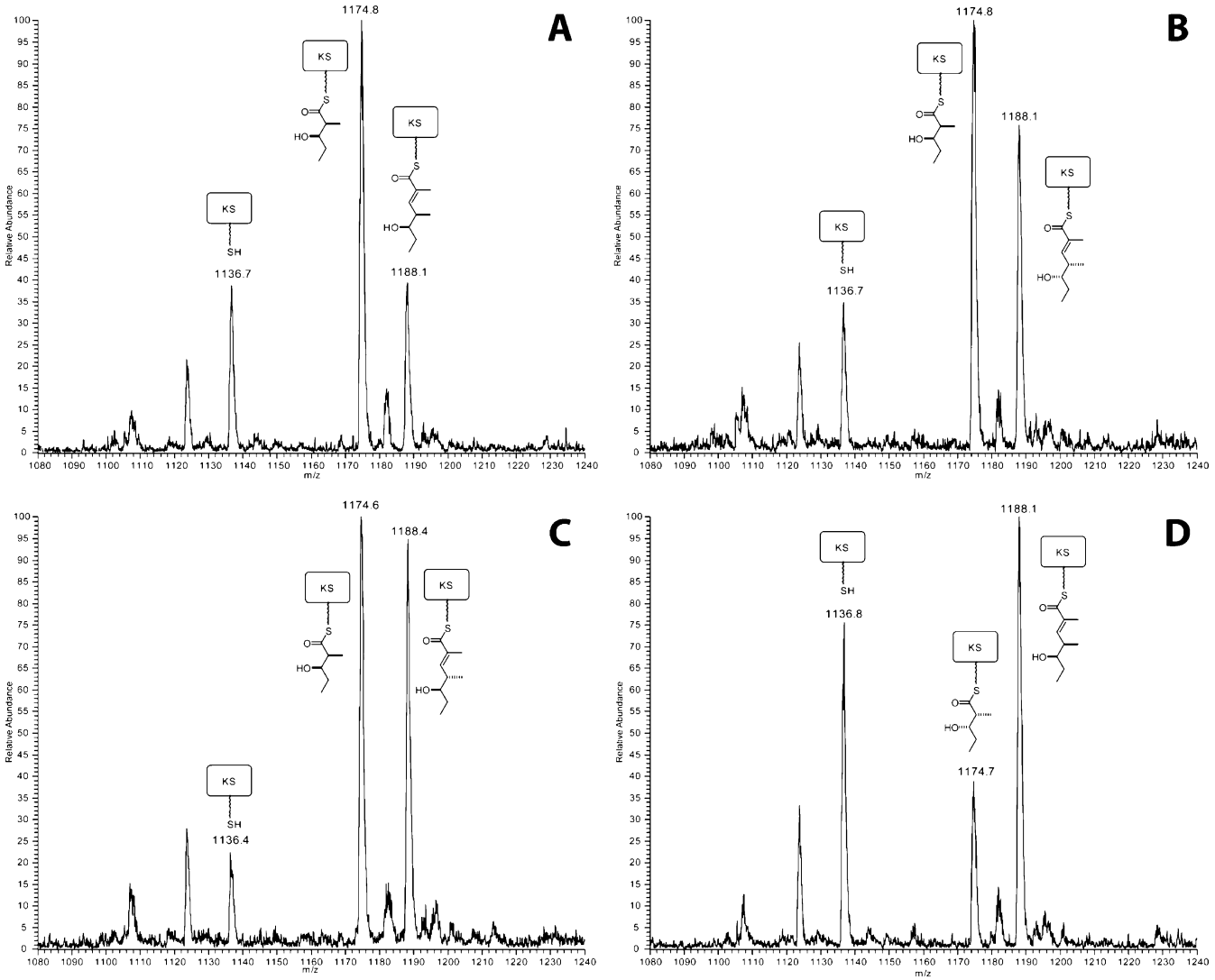

FIGURE 4: Summary of ketosynthase acylation experiments. Competitive KS loading between (A) triketide **3** and diketide **1**, (B) triketide **4** and diketide **1**, (C) triketide **5** and diketide **1**, and (D) triketide **3** and diketide **2**. ES-MS peaks corresponding to $z = +3$ are shown.

Kinetic Evaluation of Substrate Elongation. Having demonstrated the ability to detect KS-bound substrates, we addressed the challenge of monitoring KS-catalyzed chain elongation in the presence of methylmalonyl-ACP. Functional acyl carrier proteins must be covalently modified with a phosphopantetheine cofactor (*holo* form) which serves as the point of attachment for polyketide intermediates. Trypsin proteolysis of recombinant *holo*-ACP from module 3 yielded the peptide fragment AFSELGLDS*LNAMALR (MW = 2047 with a phosphopantetheine group attached to S*), as confirmed by LC-MS (see Supporting Information). Although the KS and ACP domains of an intact module are intrinsically present in an equimolar ratio, the use of discrete [KS][AT] and ACP proteins allows one to carry out multiple turnover experiments using the ACP in excess. The rate of loading of the ACP with methylmalonyl-CoA is rapid compared to the rate of KS acylation by electrophilic substrates, as determined from experiments carried out in the absence of diketide (data not shown). The observed rate of polyketide chain elongation by the [KS][AT] didomain is therefore unlikely to be measurably affected by the rate of acylation of the ACP.

Using a [KS][AT] to ACP ratio of 1:4, we examined simultaneous KS loading and substrate elongation of the previously examined series of diketide and triketide substrates. Following incubation of [KS][AT] for 1 h with **1**, methylmalonyl-CoA and *holo*-ACP were added and allowed to react for 5, 15, or 30 min. Direct proteolysis of the mixture and LC-MS analysis provided readily identified peptide fragment peaks corresponding to the KS and ACP domains at various stages of polyketide chain extension (Figure 5).

To validate the reliability of the proteolysis/LC-MS method, we also determined the kinetics of turnover of diketide **1** by traditional radio-TLC methods. Condensation of **1** with [^{14}C]methylmalonyl-CoA affords ACP-bound β-ketotriketide, which is readily excised from the enzyme by alkali-mediated lactonization. Although the overall biphasic kinetics was evident using both experimental protocols, the radiochemical experiments showed a systematic excess of roughly 0.5 turnover over the LC-MS method (Figure 6). The discrepancy was shown to be due to nonenzymatic lactonization of ACP-bound triketide product (see Supporting Information).

Compounds **2**−**5** were each assayed for relative rates of elongation, compared to **1**. As summarized in Figure 7, all substrates displayed biphasic elongation kinetics, with the initial burst phase corresponding to the first turnover. Since the reaction is initiated using [KS][AT] that has been

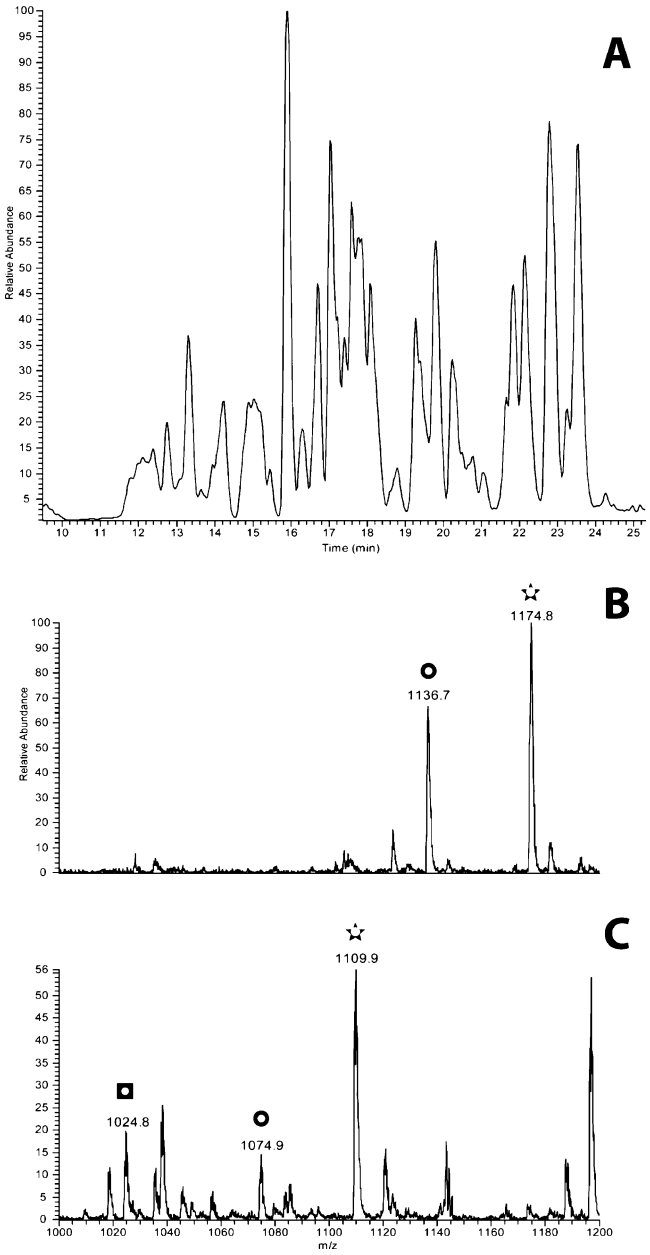

FIGURE 5: Elongation reaction with compound **1**. (A) Total ion count profile for trypsin proteolysis products from [KS][AT] and *holo*-ACP. (B) Electrospray mass spectrum [23−24 min from (A)] of $z = +3$ for unloaded KS (circle) and KS loaded with **1** (star) after one 30 min elongation reaction. (C) Electrospray mass spectrum [20−22 min from (A)] of $z = +2$ for unloaded *holo*-ACP (square), methylmalonyl-loaded *holo*-ACP (circle), and triketide elongation product on *holo*-ACP (star) after 30 min reaction time.

preacylated by diketide or triketide substrate, the biphasic kinetics reflect a change in the rate-limiting step, wherein the first turnover is limited by the kinetics of chain elongation, while subsequent turnovers are limited by the rate of reacylation of the KS domain. A critical advantage that the current methodology has over traditional radiochemical tracer techniques is that both acylation and elongation can be directly monitored simultaneously. Specifically, the level of acylation of the ketosynthase remains reasonably constant (∼55% for **1**; see Supporting Information) throughout the slower, steady-state phase of the biphasic elongation reaction. Detailed rapid-quench kinetics will be required to definitively establish the underlying mechanism for biphasic behavior.

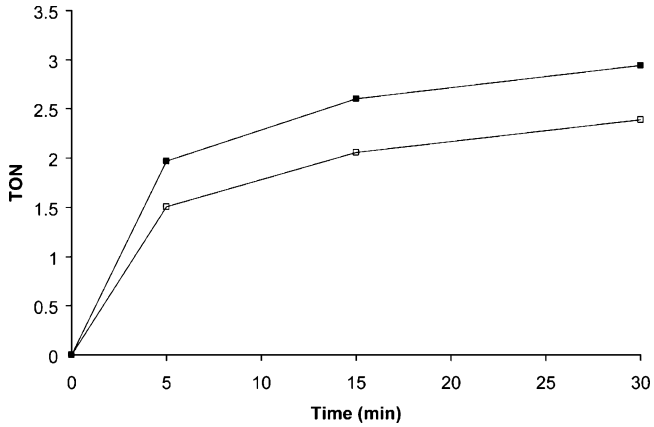

FIGURE 6: Comparison of elongation kinetics of diketide **1** by proteolysis/mass spectrometry (open squares) and radiolabeling studies (closed squares). TON = turnover number.

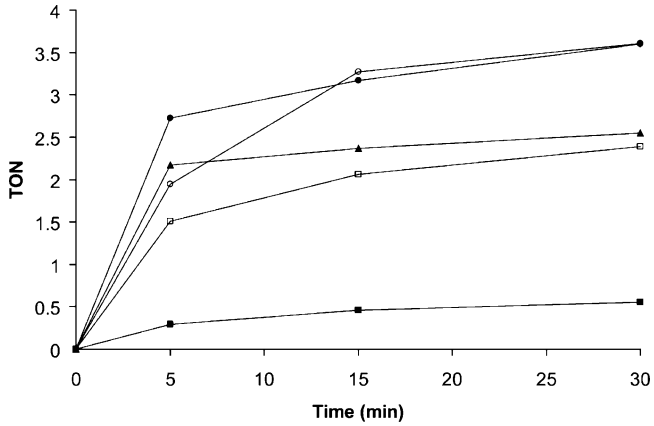

FIGURE 7: Kinetic analysis of elongation of compounds **1**−**5** with [KS][AT] and *holo*-ACP. Time points were taken at 5, 15, and 30 min for compounds **1** (open squares), **2** (closed squares), **3** (triangles), **4** (closed circles), and **5** (open circles). TON = turnover number.

Intriguingly, all three triketides exhibit modestly increased initial rates of elongation relative to **1** (Figure 7). However, despite significant differences in rates of KS acylation between triketide substrates, little variation is observed in relative rates of elongation. The (2*R*,3*S*)-diketide **2** is a poor substrate for both the KS-catalyzed acylation and the subsequent elongation reaction.

DISCUSSION

The ability to express catalytically active recombinant DEBS [KS][AT] didomains has opened up promising new avenues to probe polyketide synthase structure and function. Among the most important of the advantages is the ability to carry out multiple turnover experiments with discrete ACP domains in the absence of the thioesterase domains that have heretofore been necessary to release covalently bound products. Using mass spectrometric techniques to probe the resultant products can provide a wealth of information regarding the identity and specific location of covalently bound intermediates, as well as measurements of their relative levels as a function of substrate structure and time. Limited only by molecular mass, the method outlined in this report may permit direct discrimination of subtle mechanistic details in a variety of related biosynthetic systems.

The simple substrates utilized in this study exhibit important differences in structure, stereochemistry, and chain

length. As expected, diketide **1** acylates the KS domain significantly faster than the enantiomeric diketide **2**. It was less clear, a priori, how the remote stereocenters of the three triketides would affect acylation. The stereochemistry at C-4 and C-5 of unsaturated triketide **3** most closely resembles that of the natural substrate of DEBS module 3, yet shows the slowest rate of KS acylation of the three diketides tested. Previous attempts to generate the natural triketide thioester for module 3 resulted in rapid, non-enzyme-catalyzed lactonization of the 5-hydroxyheptanoyl-SNAC substrate.

To extend our studies to examine the substrate dependence of the second half of the KS-catalyzed reaction sequence, polyketide chain elongation by decarboxylative condensation of the methylmalonyl-S-ACP with the KS-bound acylthioester, we included the appropriate ACP domain from module 3 and analyzed the products by proteolysis/LC-MS. The [KS][AT] fragment was preloaded with the substrate of interest prior to addition of the acyl carrier protein. The extent of elongation was evaluated after 5, 15, and 30 min, with a maximum observed turnover number of 3.5 for triketides **4** and **5** (Figure 6). The apparent lower rate of elongation of diketide **1** compared to the three triketides is probably an artifact of spontaneous cyclization and release from the ACP of the β-ketotriketide lactone product derived from **1** (Supporting Information, Figure S4). Our findings suggest that, whereas considerable differences can be observed between acylation kinetics of various substrates on the KS domain of DEBS module 3, the relative rates of elongation of substrates that acylate well are similar.

In accord with previous kinetic studies involving DEBS module 2, the specificity for any given substrate, measured as the k_{cat}/K_m, is determined solely by the rate of acylation of KS since this step has been shown to be effectively irreversible (*21*). The competitive acylation experiments described, which give relative rates of KS acylation, therefore should reflect the relative k_{cat}/K_m values for compounds **1**−**5**. The proteolysis/LC-MS method is a powerful, yet complementary alternative to the use of radioactive substrates. The MS method can provide direct information about sites of protein modification as a function of substrate structure and reaction time, while the radiochemical assay will provide kinetic data of greater intrinsic accuracy.

The biphasic nature of the kinetic profiles for the substrates tested reflects a shift in the rate-limiting step after the first turnover from chain elongation to KS acylation. Under conditions where the KS domain is preloaded with the compound of interest, the observed rate for the first turnover is solely dependent on substrate elongation, given that acylation of the ACP domain by methylmalonyl-CoA is probably 1−2 orders of magnitude more rapid. Subsequent turnovers, however, require regeneration of the acylated KS prior to elongation. As acylation is significantly slower than elongation, the overall reaction rate is decreased accordingly and the latter phase of the kinetic profile is observed. Future work will require more rapid quenching techniques to determine the rate for the first turnover.

In conclusion, we have developed a tandem proteolysis/mass spectrometry procedure for direct visualization of enzyme-bound polyketide intermediates. The method has been used to monitor both ketosynthase loading and substrate elongation. An array of small molecules can be promptly surveyed to generate important information about enzyme specificity and mechanism. The method does not require substrate modifications, such as radiolabeling or florescence labeling, and structurally similar molecules can be simultaneously assayed given distinct molecular masses. Efforts to expand this approach to include redox enzymes and multimodular systems are ongoing.

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SUPPORTING INFORMATION AVAILABLE

Mass spectral data from initial proteolytic fragment identification and kinetic analysis of elongation and nonenzymatic lactonization data. This material is available free of charge via the Internet at http://pubs.acs.org.

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