Quantitative Analysis of Loading and Extender Acyltransferases of Modular Polyketide Synthases†

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ABSTRACT: The acyltransferase (AT) domains of modular polyketide synthases (PKSs) are the primary determinants of building block specificity in polyketide biosynthesis and are therefore attractive targets for protein engineering. Thus far, investigations into the fundamental biochemical properties of AT domains have been hampered by the inability to produce these enzymes as self-standing polypeptides. Here we describe an alternative, generally applicable strategy for overexpression and analysis of AT domains from modular PKSs as truncated didomain proteins (∼60 kDa). Recently, we reported the expression and reconstitution of the loading didomain of 6-deoxyerythronolide B synthase (Lau, J., Cane, D. E., and Khosla, C. (2000) *Biochemistry 39,* 10514−20). By replacing the AT domain of this protein with a methylmalonyl-CoA specific AT domain from module 6 of the 6-deoxyerythronolide B synthase, or alternatively a malonyl-CoA specific AT domain from module 2 of the rapamycin synthase, each of these extender unit AT domains could be overproduced and purified to homogeneity. Using acyl-CoA substrates as acyl group donors and *N*-acetylcysteamine as the thiol acceptor, we devised a steady-state kinetic assay to probe the properties of these three didomain proteins and selected mutants. Propionyl-CoA was the preferred substrate of the loading didomain, although acetyl- and butyryl-CoA were also accepted with ∼40-fold-lower specificity. In contrast to the relatively relaxed specificity of the loading AT domain, the methylmalonyl- and malonyl-specific AT domains had high specificity (>1000-fold) toward their natural substrates. The acyl transfer reaction was inhibited by coenzyme A (CoASH) with both a competitive and a noncompetitive component. Use of an exogenous *holo*-acyl carrier protein (ACP) as an acceptor thiol did not increase the rate of acyl transfer relative to the reaction involving *N*-acetylcysteamine, suggesting that either the on-rate of the acyl group is rate-limiting or that the *apo*-ACP component of the didomain protein precludes effective docking of a second ACP onto the AT active site. Mutation of Trp-222 in the loading AT domain to an Arg residue that is universally conserved in all extender unit AT domains failed to enable the loading AT domain to accept methylmalonyl-CoA as an alternative substrate. In contrast, mutation of the equivalent Arg residue in an extender AT domain resulted in a protein with no activity. Together, these results provide a foundation for future structural and mechanistic investigations into the properties of AT domains of modular PKSs.

Polyketide synthases (PKSs)[1] are complex multienzyme assemblies that produce polyketides, a pharmacologically important class of natural products. Despite the enormous structural diversity of polyketides, the catalytic cycle of each PKS is remarkably selective and utilizes a prescribed set of primer and extender units from the intracellular pool of acyl-CoAs. Previous studies have implicated acyltransferase (AT) domains as the primary determinants of substrate specificity (*1*−*3*). These AT domains were originally identified by homology to malonyl-CoA:ACP malonyltransferases (MATs) from fatty acid synthases, which catalyze acyl transfer via an acyl-O-enzyme intermediate involving a serine residue at the active site (Figure 1). The AT domains of PKSs share this catalytic mechanism but have variable specificity for alternative acyl-CoA substrates. Two prototypes of AT domains have been identified in modular PKSs: loading AT domains (AT_L) specify the primer unit, whereas extender AT domains control extender unit specificity. For example, the 6-deoxyerythronolide B synthase (DEBS) has a loading AT domain with specificity for propionyl-CoA and six elongation modules, each containing an AT domain that is specific for (2*S*)-methylmalonyl-CoA.

Efforts to rationally modify polyketide structure by replacing naturally occurring AT domains in modules with heterologous domains have borne considerable success (*4*−*10*). For example, substitution of a malonyl-specific AT domain from the rapamycin polyketide synthase (RAPS) into DEBS

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[1] Abbreviations: AT, acyltransferase; PKS, polyketide synthase; HSNAc, *N*-acetylcysteamine; CoASH, coenzyme A; ACP, acyl carrier protein; MAT, malonyl-CoA:ACP malonyltransferase; DEBS, 6-deoxyerythronolide B synthase; RAPS, rapamycin synthase; LDD, loading didomain; TLC, thin-layer chromatography; HPLC, high-performance liquid chromatography.

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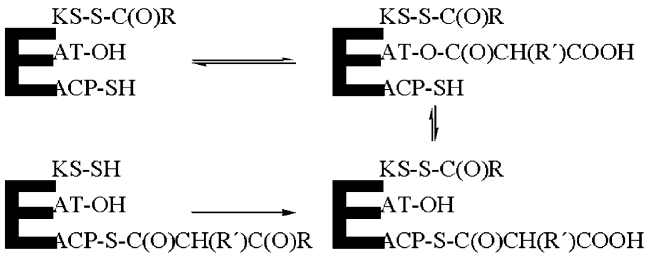

FIGURE 1: Representation of a minimal elongation module of a polyketide synthase, which consists of a ketosynthase (KS), an acyltransferase (AT), and an acyl carrier protein (ACP) domain. The growing chain, R, is attached to the KS. An acyl unit, C(O)-CH(R′)COOH, is transferred to the ACP via an acyl-O-AT intermediate. Finally, through a decarboxylative condensation reaction, the acyl unit is joined to the growing chain and passed back to the KS domain and is thus ready for another cycle of elongation.

yields regioselectively desmethylated analogues of 6-deoxyerythronolide B (or erythromycin). More recently, the same goal has also been achieved by site-directed mutagenesis of selected residues in a targeted AT domain (*11*). Notwithstanding these successes, little is known about the fundamental properties of AT domains both as stand-alone catalysts and in the context of PKS modules, principally because of the difficulties associated with analyzing their catalytic activities in isolation from the rest of the megasynthase.

Previous attempts to express functional AT domains as intact proteins have not been successful, in most cases yielding insoluble inclusion bodies (unpublished results). One exception was the functional expression of the DEBS loading didomain (LDD), comprised of the propionyl-specific AT loading domain covalently linked to an acceptor acyl carrier protein (ACP) domain (*12*). Coexpression of LDD in the absence or presence of a phosphopantetheinyl transferase, Sfp, allowed for production of the apo or holo forms of the protein. Availability of these proteins facilitated preliminary investigations into the properties of the loading AT, which demonstrated that this AT has relaxed specificity toward a variety of primer units.

Here, we continue our investigations into the properties of individual AT domains. The expression system for the LDD protein has been modified to functionally overexpress methylmalonyl- and malonyl-specific AT domains. By analogy with Type II fatty acid synthases (*13*−*17*) where the MAT is expressed as a distinct polypeptide, two types of multiple turnover assays were developed for both loading and extender AT domains of modular PKSs. In one assay, the acyl group is transferred from the corresponding acyl-CoA to a *N*-acetylcysteamine (HS-NAc) thiol acceptor (Figure 2). These acyl-S-NAc products can then be readily quantified via radio-TLC (thin-layer chromatography) or HPLC (high-performance liquid chromatography). Alternatively, an exogenous, recombinant *holo*-ACP from a modular PKS was used as the thiol acceptor. Acyl-ACPs could then be quantified by TCA precipitation and liquid scintillation counting. Together, the novel expression system and assay formats have been used to quantify and compare the substrate specificity of the loading AT domain of DEBS, a methylmalonyl-specific extender AT domain from DEBS, and a malonyl-specific extender AT domain from the rapamycin synthase.

FIGURE 2: Schematic of the HS-NAc assay. An acyl-S-CoA is incubated with HS-NAc and a didomain protein in which the ACP is expressed in an inactive apo form. The AT domain catalyzes the transfer of a radioactive acyl group from CoA to HS-NAc.

EXPERIMENTAL PROCEDURES

Materials. TLC plates (IB2-F) and silica gel (40 μm) were from J. T. Baker. [1-^{14}C]Propionyl-CoA (55 mCi/mmol) and [1-^{14}C]acetyl-CoA (50 mCi/mmol) were from Moravek Biochemicals; [1-^{14}C]butyryl-CoA (4 mCi/mmol) and [2-^{14}C]-malonyl-CoA (58.7 mCi/mmol) were from NEN Life Sciences; DL-[2-^{14}C]methylmalonyl-CoA (60 mCi/mmol) was from American Radiolabeled Chemicals; and [9,10(*n*)-^{3}H]-myristoyl-CoA was from Amersham Pharmacia Biotech. *N*-acetylcysteamine (HS-NAc) was purchased from Aldrich. All other chemicals, including CoA derivatives, were from Sigma and of the highest available purity. Restriction enzymes were from New England Biolabs.

Manipulation of DNA and Strains. DNA manipulations were performed in *Escherichia coli* XL1-Blue (Stratagene) using standard culture conditions (*18*). Polymerase chain reactions (PCR) were carried out using Pfu polymerase (Stratagene) as recommended by the manufacturer. Mutants were generated using the QuikChange Site-Directed Mutagenesis Kit (Stratagene).

Construction of an Alternative Expression Vector for the DEBS Loading Didomain. Plasmid pGFL34b is a derivative of pJL636 (*12*) in which the *NdeI*−*EcoRI* fragment was transferred to a pET28c vector (Novagen), thus resulting in an expression vector with hexahistidine tags appended to both the N- and C-termini.

Construction of Expression Vectors for Extender AT Domains. Figure 3A summarizes the overall topology of didomain proteins. Plasmid pJL663 is a derivative of pJL636 (*12*) in which a 944-bp *MscI*−*PstI* fragment corresponding to the pAT_L domain was replaced with a 935-bp *MscI*−*PstI* fragment corresponding to the mmAT-6 domain of DEBS. This plasmid was constructed as follows. A PCR-amplified *ClaI*−*MscI* fragment (nucleotides 718−926; GenBank accession no. M63676) corresponding to the N-terminus of DEBS1 was introduced into a subclone containing a PCR-amplified *MscI*−*NheI* fragment extending from nucleotides 16 785−17 589 of the DEBS mmAT-6 domain (GenBank accession no. M63677) to form plasmid pJL668. Concurrently, a PCR-amplified *NheI*−*StyI* fragment extending from nucleotides 17 589−17 679 of the DEBS mmAT-6 domain was ligated to a PCR-amplified *StyI*−*PstI* fragment extending from nucleotides 17 679−17 720. The resulting 131-bp *NheI*−*PstI* fragment was then introduced into a subclone containing a PCR-amplified *PstI*−*EcoRI* fragment corresponding to the ACP_L domain of DEBS (nucleotides 1870−2228; GenBank accession no. M63676) to generate plasmid pJL669. The 1012-bp *ClaI*−*NheI* and 489-bp *NheI*−*EcoRI* fragments from pJL668 and pJL669, respectively, were

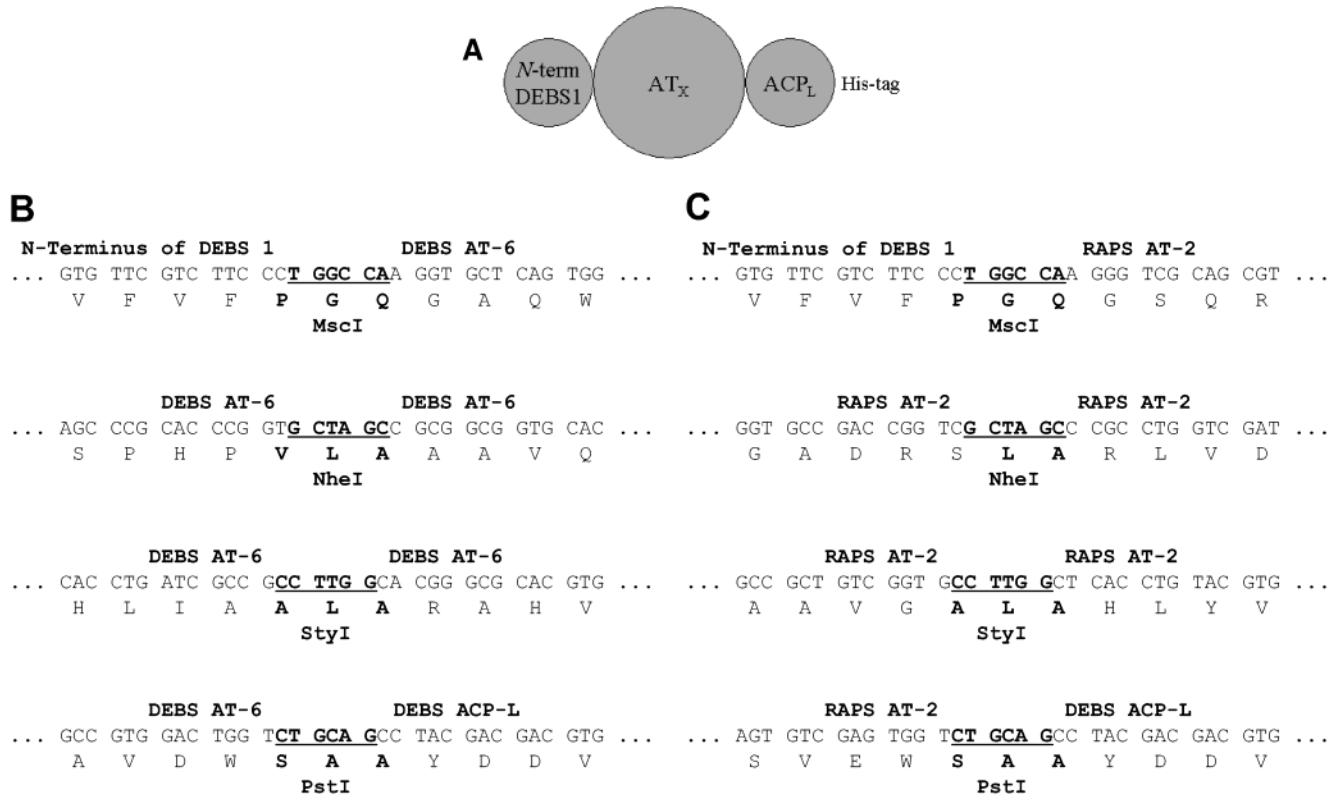

FIGURE 3: (A) Schematic of resulting didomain proteins in which X = DEBS AT-loading, DEBS mmAT-6, or RAPS mAT-2. Nucleotide sequence at the engineered restriction sites of the extender didomains. (B) Substitution of the AT$_L$ domain of DEBS with the mmAT-6 domain of DEBS (pJL663). (C) Substitution of the AT$_L$ domain of DEBS with the mAT-2 domain of RAPS (pJL666).

subsequently excised and introduced into *ClaI*−*EcoRI*-digested pJL636 by a three-fragment ligation to produce the expression plasmid pJL663. The engineered boundaries for this extender didomain are shown in Figure 3B.

Analogous to plasmid pJL663, plasmid pJL666 is a derivative of pJL636 (*12*) in which the 944-bp *MscI*−*PstI* fragment corresponding to the pAT$_L$ domain was replaced with an 824-bp *MscI*−*PstI* fragment corresponding to the mAT-2 domain of RAPS. This plasmid was constructed as follows. A PCR-amplified *MscI*−*NheI* fragment from the RAPS mAT-2 domain (complement of nucleotides 54 822−55 536; GenBank accession no. X86780) was ligated into *MscI*−*NheI*-digested pJL668 to form pJL670. At the same time, a *NheI*−*StyI* fragment (complement of nucleotides 54 754−54 822) and a *StyI*−*PstI* fragment (complement of nucleotides 54 712−54 754) from the RAPS AT-2 domain were amplified by PCR, and these were ligated together. The resulting 110-bp *NheI*−*PstI* fragment was then introduced into *NheI*−*PstI*-digested pJL669 to form pJL671. The 922-bp *ClaI*−*NheI* and 468-bp *NheI*−*EcoRI* fragments from pJL669 and pJL671, respectively, were subsequently excised and introduced into *ClaI*−*EcoRI*-digested pJL636 by a three-fragment ligation to produce the expression plasmid pJL666. The engineered boundaries for this extender didomain are shown in Figure 3C.

Expression and Purification of N- and C-Terminally His-Tagged Loading Didomains. To produce the apo form of the DEBS loading didomain, plasmid pGFL34b was introduced into *E. coli* BL21(DE3) cells (Stratagene) via transformation. One-liter cultures were grown at 37 °C in 2-L flasks containing Luria−Bertani broth supplemented with 50 μg/mL kanamycin. At an OD = 0.6, the culture was

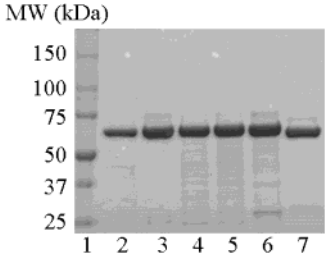

FIGURE 4: Purity of recombinant proteins purified from *E. coli*. Protein samples were resolved by Tris-HCl SDS−PAGE (4−15%) and stained with Coomassie blue. Lane 1, molecular weight markers; 2, *apo*-LDD; 3, *holo*-LDD; 4, W222A LDD; 5, W222R LDD; 6, DEBS mmAT-6 didomain; and 7, RAPS mAT-2 didomain.

transferred to a temperature of 21 °C, induced with 50 μM IPTG, and grown overnight. Cells were harvested by centrifugation, and the pellet was then resuspended in 25 mL of 50 mM Tris (pH 8.0) and 5 mM imidazole. Cells were disrupted by three passes through a French press or by sonication, and the supernatant was batch-loaded onto nickel-nitrilotriacetic acid agarose (Qiagen). The resin was loaded onto a column and washed with 50 mM Tris (pH 8.0) and 50 mM imidazole. The protein was eluted with 50 mM Tris (pH 8.0) and 100 mM imidazole and concentrated in a Centriprep-30 concentrator (Amicon). Protein concentration was estimated using the Lowry method (Sigma). A typical 1-L culture produced about 20 mg of purified enzyme. *Holo*-LDD and mutants of the loading AT domain were expressed and purified by the same procedure and produced comparable amounts of protein (Figure 4).

Overexpression and Purification of Extender Didomain Proteins. For expression of apo extender didomains, plasmids

pJL663 and pJL666 were independently introduced into *E. coli* BL21(DE3) (Stratagene) via transformation. One-liter cultures of these recombinant *E. coli* strains were grown at 37 °C in 2-L flasks containing LB medium supplemented with 100 μg/mL carbenicillin. The expression and purification steps for these proteins were performed in the same manner as described for the loading didomain of DEBS (*12*). Protein concentrations were determined using the Lowry method (Sigma). A typical 1-L culture produced about 8 mg of purified enzyme (Figure 4).

Construction of Loading Didomain Mutant Plasmids (pGFL36 W222A and pGFL37 W222R). Plasmids pGFL36 and pGFL37 were created according to manufacturer's instructions for the QuikChange Site-Directed Mutagenesis Kit. The following pairs of oligos were used: 5′-GCCGCCGCGCTG**GCG**AGCCGCGAGATGATTCC-3′ and 5′ GGAATCATCTCGCGGCT**CGC**CAGCGCGGCGGC-3′; 5′-GCCGCCGCGCTG**CGG**AGCCGCGAGATGATTCC-3′ and 5′-GGAATCATCTCGCGGCT**CCG**CAGCGCGGCGGC-3′; and pJL636 was used as the template (*12*). After the mutations were verified by sequencing, the *NdeI*−*EcoRI* fragments of these two mutants were then subcloned into pET28c (Novagen).

Construction of Extender Didomain Mutant Plasmids (pGFL45 R224A and pGFL46 R224W). Plasmids pGFL45 and pGFL46 were created according to manufacturer's instructions for the QuikChange Site-Directed Mutagenesis Kit. The following pairs of oligos were used: 5′-CGCGTCGTGGCCCTG**GCC**GCGAAGGCGTTGCG-3′ and 5′- GCCAACGCCTTCGC**GGC**CAGGGCCACGACGCG-3′; and 5′-CGCGTCGTGGCCCTG**TGG**GCGAAGGCGTTGCG-3′ and 5′- GCCAACGCCTTCGC**CCA**CAGGGCCACGACGCG-3′. The *NdeI*−*EcoRI* fragment was subcloned into pBluescript, which was used as the template for the mutation reaction. After the mutations were sequenced, the *NdeI*−*EcoRI* fragment was then transferred into pET21c (Novagen).

Radioactive Labeling of Proteins. A 15 μM protein sample was incubated with 75 μM radiolabeled substrate in a volume of 10 μL for 5 min on ice. The reaction was quenched by the addition of Laemmli buffer (lacking reducing agent). The entire mixture was loaded immediately on a 4−15% gradient Tris-HCl gel (Bio-Rad) and separated via electrophoresis. The gel was vacuum-dried, then exposed to X-ray film and developed.

Quantification of Substrate Selectivity Using HS-NAc as the Thiol Acceptor. As described above, the didomain proteins contain an AT domain (~40 kDa) and an ACP domain (~10 kDa). To facilitate interrogation of the properties of the AT domains alone, the ACP domain of these didomain proteins was expressed in an inactive apo form. Without the functionality gained by the addition of a phosphopantetheine arm to the ACP domain, the didomain protein acts simply as an isolated AT domain, allowing us to probe the intrinsic specificity of the AT domain. In place of the phosphopantetheinyl thiol of *holo*-ACP in the natural reaction, *N*-acetylcysteamine (HS-NAc) serves as the thiol acceptor in the transacylation reaction (Figure 2). Kinetic assays were performed using radioactive acyl-CoA substrates, an apo didomain protein, a HS-NAc thiol acceptor in 100 mM NaH_2PO_4, pH 7.2 or 7.0 (pH was lowered for slower enzymes to minimize the background (nonenzymatic) rate of acyl transfer), 2.5 mM DTT, 1 mM EDTA, and 20% glycerol in typical reaction volumes of 10−40 μL. Proteins were assayed at room temperature unless otherwise noted. Reactions were quenched by the addition of 20 μL of ice-cold acetone, applied to a TLC plate, and developed in 89% ethyl acetate, 10% 2-propanol, and 1% trichloroacetic acid. The plate was imaged and quantified by autoradiography (Packard Instant Imager 2024). A typical TLC autoradiograph is shown in Figure 5. For ^{3}H-labeled myristoyl-CoA, the product was separated via TLC and counted by liquid scintillation counting (Beckman LS 3801).

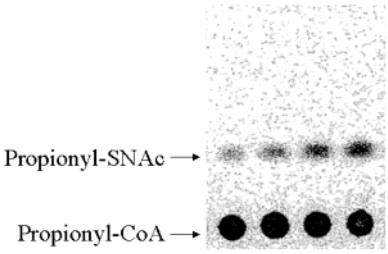

FIGURE 5: Typical HS-NAc time course analyzed on a TLC autoradiograph, showing the baseline spot (propionyl-CoA) and propionyl-SNAC. Reaction shown is 0.1 μM *apo*-LDD, 7.5 μM propionyl-CoA, and 10 mM HS-NAc at 0.5, 1, 1.5, and 2 min time points.

HPLC Analysis of HS-NAc Assay. In some cases, the AT-catalyzed reactions involving acyl-CoA and HS-NAc substrates were analyzed via HPLC. Assays were performed as previously described and quenched in ice-cold acetone (1% trichloroacetic acid). Reactions were passed through a 1-mL silica flash column to remove unreacted acyl-CoA. Acyl-S-NAc compounds were eluted with 1 mL of 89% ethyl acetate, 10% 2-propanol, and 1% trichloroacetic acid. The flow-through and eluate were collected and vacuum-dried. The product was resuspended in 20 μL of a 50:50 methanol/water mixture and injected onto a C_{18} reverse-phase HPLC column (VYDAC, 250 × 5 mm) with a nearly isocratic gradient: Buffer A = water + 0.1% TFA and Buffer B = acetonitrile + 0.1% TFA. A 10-min gradient between 50 and 52% Buffer B was used. Individual fractions were collected every 15 s. An aliquot of 4 mL of scintillation fluid (Ready-Safe, Beckman) was added to the fraction and counted by liquid scintillation counting. Authentic references were from Aldrich or synthesized as described earlier (*19*). The HPLC peaks were verified by mass spectrometry. Typical HPLC graphs are shown in Figure 6A.

To assess the relative specificity of two substrates, competition assays were performed with a radiolabeled substrate and an unlabeled substrate in the same reaction. Conversion of the labeled substrate to acyl-S-NAc was followed by HPLC/ liquid scintillation counting, as described above.

Inhibition of the AT Reaction by CoASH. Reactions were performed as previously described, in the presence of 30, 70, and 100 μM CoASH.

Acyl-CoA/Holo-ACP Acyl Transfer Assay. A 20 kDa truncated ACP from module 4 of DEBS, which includes a C-terminal linker expressed from plasmid pNW8 (*20*), was purified and used as an alternative acyl acceptor. Assays contained 100 μM *holo*-ACP, 0.1 μM LDD, and 1 mM propionyl-CoA in volumes of 10 μL. Reactions were quenched in 0.5 mL of ice-cold 10% trichloroacetic acid and incubated for 30 min on ice. The precipitated protein was

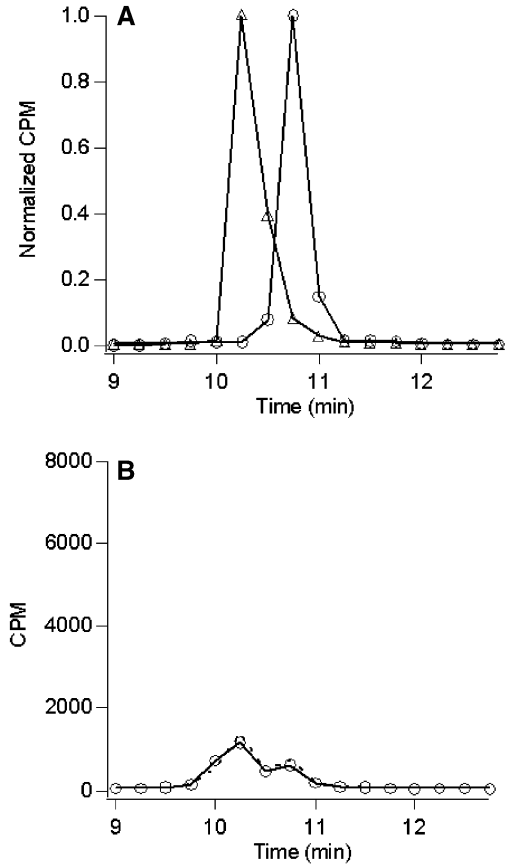

FIGURE 6: (A) Typical HPLC traces in 15-s fractions were collected and analyzed by liquid scintillation counting, showing the separation between the propionyl-SNAc (○) and the methylmalonyl-SNAc (△) peaks. (B) LDD catalysis of methylmalonyl-CoA with HS-NAc. Dashed line (−) shows nonenzymatic control. In this assay, 8000 CPM would correspond to a k_{cat}/K_M ~0.001 μM^{-1} min^{-1}.

separated by centrifugation, and the pellet was washed two more times with 0.5 mL of cold 10% trichloroacetic acid. The pellet was finally dissolved in 0.5 mL of 20 mM NaOH and 2% SDS, added to 4 mL of scintillation fluid (Ready-Safe, Beckman), and counted by liquid scintillation counting (Beckman LS 3801).

RESULTS

Expression and Purification of N- and C-His-Tagged Loading Didomain. The addition of an N-terminal His-tag resulted in a 20-fold increase in protein production over the original C-terminally His-tagged construct (*12*). Not surprisingly, the dual-tagged proteins bound more tightly to the Ni-NTA resin and therefore simplified the purification to a single-step procedure. Figure 4 shows the purified protein. The dual His-tagged protein was comparably active to the single His-tagged protein (data not shown).

Construction and Purification of Extender Didomains. Earlier attempts to express individual AT domains from DEBS as monodomain proteins were unsuccessful, resulting predominantly in the production of inclusion bodies in *E. coli*. Motivated by our success in overexpression and purification of the loading didomain of DEBS as an isolated protein (*12*) and by its relatively small size (60 kDa), we attempted to replace the AT domain in that construct with other AT domains from chain extension modules of DEBS

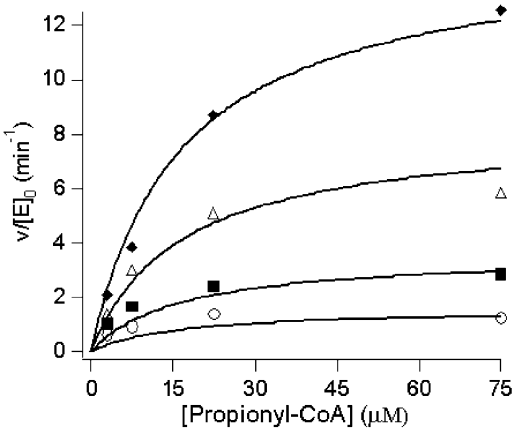

FIGURE 7: Representative kinetic data for LDD at 0.4 mM (○), 1 mM (■), 3 mM (△), and 10 mM (◆) HS-NAc. Plotted is a representative plot of the rate of formation of propionyl-S-NAc divided by the enzyme concentration, 0.1 μM LDD. Also shown is the fit to a two-substrate Michaelis−Menten equation. See Experimental Procedures for details.

and other PKSs. Specifically, two extender didomain constructs were engineered by independently replacing the loading AT_L of the AT_L−ACP_L protein with a methylmalonyl-specific AT domain from module 6 of DEBS (hereafter referred to as DEBS mmAT-6) and a malonyl-specific AT domain from module 2 of the rapamycin polyketide synthase (hereafter referred to as RAPS mAT-2). Both extender didomains were expressed in *E. coli* BL21(DE3) as C-terminal hexahistadine $(His)_6$-tagged fusion proteins. They were purified by nickel affinity chromatography followed by anion-exchange chromatography to >95% homogeneity (Figure 4). On the basis of dynamic light scattering (DLS) experiments, both the DEBS mmAT-6 and the RAPS mAT-2 didomains behaved as dimers with a molecular weight of approximately 140 kDa (data not shown). This finding is consistent with the dimeric nature of the DEBS proteins (*21*−*23*).

HS-NAc Assay. TLC and HPLC were used to monitor the enzyme-catalyzed formation of acyl-S-NAc from the corresponding acyl-CoA and HS-NAc. The authenticity of products of the enzymatic reactions was also confirmed by extraction from the TLC plate, followed by mass spectrometry (data not shown). Radio-TLC procedures could be used to detect as little as 1 ± 0.2 pmol product, whereas HPLC analysis could reliably detect 0.5 ± 0.05 pmol product. In some cases (e.g., assays involving DEBS mmAT-6) where turnover numbers were relatively low, the background rate of acyl transfer contributed to >10% of the product formation. In such circumstances, the pH of the assay buffer was adjusted to 7.0 to reduce nonenzymatic transacylation to <10%.

The steady-state kinetic parameters of the loading didomain were measured via a 4 × 4 plot by varying the concentrations of both substrates, propionyl-CoA, and HS-NAc. The experiment was repeated three times, and the data were fit to a two-parameter Michaelis−Menten equation, resulting in a $k_{cat} = 27 \pm 5$ min^{-1}, $K_{M,propionyl-CoA} = 14 \pm 2$ μM, and $K_{M,HS-NAc} = 4 \pm 2$ mM (Figure 7). To quantify the extent of inhibition of the AT-catalyzed reaction by CoASH, the rate of propionyl transfer was measured in the presence of varying amounts of CoASH, and the data from two independent experiments were fit to a mixed inhibition

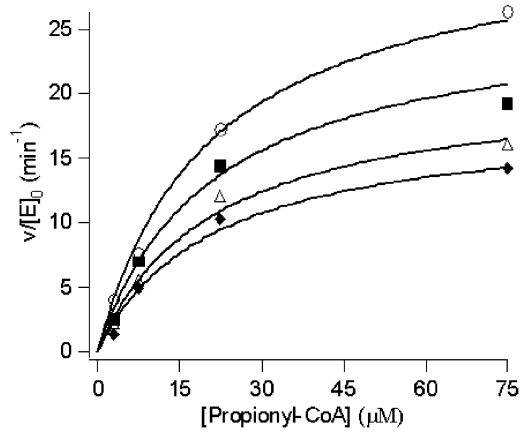

FIGURE 8: Inhibition of transacylation reaction by 0 μM (○), 30 μM (■), 70 μM (△), and 100 μM (◆) free CoA. Reaction was performed with 10 mM HS-NAc and 0.1 μM LDD as a function of propionyl-CoA concentration. Shown is the fit to a mixed inhibition system. See Experimental Procedures and Results for details.

model, resulting in a competitive K_i of ~200 μM with a noncompetitive component K_i of ~100 μM (Figure 8).

The steady-state kinetic properties of the extender unit AT domains, DEBS mmAT-6 and RAPS mAT-2, expressed in the didomain format described above were also analyzed using their natural substrates, methylmalonyl-CoA and malonyl-CoA, respectively. Remarkably, neither enzyme showed evidence of saturation at substrate concentrations less than 100 μM (data not shown); therefore, only the k_{cat}/K_M parameters could be accurately determined. To verify that HS-NAc was saturating, the rates of transacylation catalyzed by DEBS mmAT-6 were measured at 10 and 30 mM HS-NAc in the presence of 100 μM methylmalonyl-CoA. Within error, these rates were indistinguishable (data not shown), suggesting that the enzyme was saturated with respect to HS-NAc. In the presence of 10 mM HS-NAc and 0.1 μM didomain protein, the k_{cat}/K_M of DEBS mmAT-6 for methylmalonyl-CoA at 30 °C and pH 7.0 was 0.021 ± 0.001 μM^{-1} min^{-1}, whereas that of RAPS mAT-2 for malonyl-CoA at room temperature and pH 7.2 was 0.34 ± 0.03 μM^{-1} min^{-1}.

ACP Assay. An exogenously expressed *holo*-ACP could also serve as a thiol acceptor, as described in the Experimental Procedures section. Using the ACP domain of module 4 of DEBS at a concentration of 100 μM, the rate of transfer was ~0.1 min^{-1}, either with *apo*-LDD or *holo*-LDD as the catalyst (data not shown), suggesting that exogenous HS-NAc is at least as good a substrate as an exogenous *holo*-ACP for the AT-catalyzed reaction.

Substrate Specificity of Loading and Extender AT Domains. The HS-NAc assay allowed us to make direct measurements of the specificity of some of the other acyl-CoA substrates previously tested against the loading AT of DEBS (*12*). Table 1 lists the k_{cat} and K_M as well as specificity constants, k_{cat}/K_M, for acetyl-CoA, propionyl-CoA, and butyryl-CoA. As can be seen from these data, the loading AT is more specific than was previously estimated via competition experiments in which the relative acyl group occupancy of the *holo*-AT_L−ACP_L protein at steady-state was measured by TCA precipitation. Moreover, from our measurements reported here (Table 1), specificity appears

Table 1: k_{cat}, K_M, and k_{cat}/K_M of LDD for Propionyl-, Acetyl-, and Butyryl-CoA[a]

	k_{cat} (min^{-1})	K_M (μM)	k_{cat}/K_M (min^{-1} μM^{-1})
propionyl-CoA	12.4	13.3	0.93
acetyl-CoA	2.5 ± 0.3	110 ± 10	0.024
butyryl-CoA	4.7 ± 0.2	230 ± 30	0.021

[a] Reactions were performed at pH = 7.0 to minimize background transacylation, and 1 μM enzyme was included in the reaction.

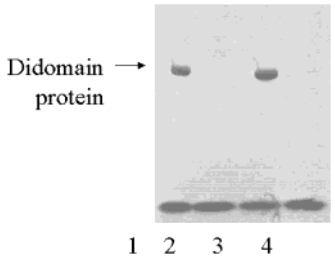

FIGURE 9: Autoradiogram of a 4−15% SDS−PAGE gel showing labeling of the purified DEBS mmAT-6 and RAPS mAT-2 didomains with both [^{14}C]methylmalonyl- and [^{14}C]malonyl-CoA. Lane 1, DEBS mmAT-6 didomain with methylmalonyl-CoA; Lane 2, DEBS mmAT-6 didomain with malonyl-CoA; Lane 3, RAPS mAT-2 didomain with malonyl-CoA; and Lane 4, RAPS mAT-2 didomain with methylmalonyl-CoA.

to be principally dictated by the K_M of the enzyme for different substrates. Finally, we attempted to directly measure the turnover number of ^{3}H-myristoyl-CoA by the loading AT domain; however, no enzymatic activity could be detected for this substrate.

Earlier studies based on competition experiments involving labeled acetyl- or propionyl-CoA and unlabeled methylmalonyl-CoA suggested that methylmalonyl-CoA may also be accepted by the loading AT domain as a substrate. To directly test this hypothesis using the new assay, HPLC analysis was performed on a reaction mixture containing radiolabeled methylmalonyl-CoA, HS-NAc, and the loading didomain. After a 5 min incubation, no radiolabel was detected as comigrating with the authentic methylmalonyl-SNAc peak (Figure 6B), suggesting that, if methylmalonyl-CoA is indeed accepted by this enzyme, its k_{cat}/K_M must be below ~0.001 μM^{-1} min^{-1}.

To evaluate the relative selectivity of DEBS mmAT-6 and RAPS mAT-2 for malonyl-CoA versus methylmalonyl-CoA, the proteins were initially incubated with low concentrations of their natural substrates (50 μM) or alternatively high concentrations (200 μM) of the other substrate and analyzed via SDS−PAGE autoradiography. As shown in Figure 9, in neither case did the unnatural substrate label the protein, suggesting that the extender unit ATs were more selective than the loading AT of DEBS. To estimate the relative specificities of DEBS mmAT-6 and RAPS mAT-2 for natural versus unnatural substrates, competition assays were performed with unlabeled natural substrate (10 μM) and competing radiolabeled unnatural substrate (200 μM). In both cases, the substrate specificity for alternative substrates was estimated by radio-HPLC to be less than 1/1000 of that of the natural substrates.

Mutagenesis of Loading and Extender Didomains. Earlier studies by Rangan and Smith (*24*) on the malonyltransferase domain of the mammalian fatty acid synthase demonstrated

the importance of a single Arg residue in discriminating between extender (α-carboxylated) units and primer (non-carboxylated) units. This Arg residue is invariably conserved in all extender unit AT domains of modular PKSs as well; however, in two loading AT domains (of DEBS and the avermectin synthase) a Trp residue is present at this position. To test whether the residue in this position is critical in discriminating between extender and primer units, the Trp residue of DEBS AT_L and the Arg residue of DEBS mmAT-6 were each mutated to the corresponding Arg and Trp residues, respectively, as well as to Ala. Mutant proteins were purified as described above, and their activities were assessed via the HS-NAc assay.

The two mutants of the loading AT, W222A and W222R, had a 10-fold decreased k_{cat} toward the natural substrate, propionyl-CoA, and no measurable activity toward the carboxylated analogue, methylmalonyl-CoA (data not shown). Two mutants of the extender DEBS mmAT-6, R224A and R224W, were also assessed. Both mutants showed no measurable activity toward either the natural substrate, methylmalonyl-CoA, or the decarboxylated substrate, propionyl-CoA, even though they purified analogous to the wild-type didomain protein on nickel affinity and HiTrap Q columns (data not shown).

DISCUSSION

Given the importance of AT domains in polyketide biosynthesis and engineering, a detailed understanding of their mechanism and specificity is desirable. Two types of AT domains are found in modular PKSs: primer unit ATs that transfer acyl chains from substrates such as acetyl-, propionyl-, and isobutyryl-CoA to a designated ACP; and extender unit ATs that transfer α-carboxylated acyl groups from substrates such as malonyl- and methylmalonyl-CoA. Early studies on the substrate specificity of the loading AT domain of DEBS (*12*, *25*) were performed on a large polypeptide that was typically available in only small quantities and catalyzed multiple reactions. In contrast, studies on the homologous aromatic Type II PKSs has allowed individual reactions to be evaluated because catalysis is carried out by separate polypeptides. The acyltransferase reaction is fast, over 400 s^{-1} (*17*), whereas the condensation reaction catalyzed by the ketosynthase has been measured to be over 4 orders of magnitude slower, 0.4 min^{-1} (*26*), at the same temperature. Experiments in our lab have also shown that chain extension is significantly slower than the acyltransferase reaction in intact Type I PKSs (Marcus Hans, submitted). Thus, the overall polyketide synthesis reaction reflects the rate-determining extension step, and a separate assay must be devised to probe acyltransferase specificity.

Recently (*12*), the AT_L-ACP_L didomain of DEBS was successfully expressed, thereby facilitating preliminary analysis of its substrate specificity. For this, an assay was used in which radiolabeled propionyl-CoA and a competing unlabeled substrate were co-incubated with the *holo*-protein. Under steady-state conditions, the decrease in labeling intensity relative to a control lacking the unlabeled substrate was assumed to correlate with the actual specificity of the loading AT domain for the unlabeled substrate. In this report, we describe the development of a direct assay for measuring AT specificity and exploit it to measure the specificity of the loading AT domain for the predominant physiological substrates available to DEBS in a typical bacterial cell. At the same time, we also report on a general method for overexpressing extender unit AT domains and use this protein engineering strategy to study a representative methylmalonyl-specific and malonyl-specific AT domain. Fusion of ACP_L to DEBS mmAT-6 and RAPS mAT-2 yields soluble protein, with the option of expressing ACP_L in an inactive (apo) or active (holo) form. Since isolated AT domains from modular PKSs do not appear to express as soluble proteins in *E. coli*, the ACP_L seems to act as a chaperone, perhaps burying an unstable hydrophobic region in the AT domains.

The AT assay described here utilizes HS-NAc as an alternative thiol acceptor. We also investigated the inhibition of the loading AT by CoASH. As expected of a ping-pong bi bi mechanism, the reaction resulted in mixed inhibition with respect to propionyl-CoA, with both competitive and noncompetitive components giving rise to K_i values on the order of 100−200 μM.

To evaluate the contribution of the thiol acceptor in the acyltransferase reaction, we also used an exogenous *holo*-ACP as an acceptor in a more reagent-intensive TCA precipitation assay. Propionyl transfer by the loading of AT to ACP-4 of DEBS could be measured, yet the turnover number was not higher than that for HS-NAc, even when adjusted for different acceptor concentrations in the two assay formats. This suggests that formation of the acyl-enzyme intermediate is likely to be the rate-limiting step in the overall catalytic cycle. Alternatively, it may highlight the potential inhibitory effect of the covalently fused ACP_L domain on the catalytic activity of the loading AT, which precludes effective docking of the exogenous ACP at the active site of the AT domain. Further kinetic and protein chemical studies could be useful in this regard.

With the HS-NAc assay system established, we also investigated the substrate specificity of the two extender AT domains. In neither case were we able to saturate the AT to calculate k_{cat} and K_M. For DEBS mmAT-6, a $k_{cat}/K_M \sim 0.021 \pm 0.001$ min^{-1} μM^{-1} was measured in the presence of 10 mM HS-NAc, whereas RAPS mAT-2 was more active with a $k_{cat}/K_M \sim 0.34 \pm 0.03$ min^{-1} μM^{-1}. The mechanistic basis for the differences in these kinetic parameters is unclear and may yet again point to the variable inhibitory role of the loading ACP on these two reactions. Alternatively, it may highlight the closer evolutionary relationship of malonyl-specific AT domains to fatty acid AT domains, which have substantially higher turnover numbers. Perhaps in evolving AT domains to accept methylmalonyl-CoA, the overall catalytic capability of this enzyme was compromised. Since acyl transfer does not limit the entire PKS reaction, such a loss of kinetic power may have been an acceptable tradeoff during the evolution of new polyketide pathways.

Although HS-NAc, a truncated form of the pantetheine arm on the ACP, is not a physiological substrate, the assay allows for a comparative analysis of specificity toward different substrates. For example, comparison of the specificity constants for propionyl-CoA, acetyl-CoA, and butyryl-CoA revealed that the specificity of the loading AT for acetyl-CoA and butyryl-CoA are similar, whereas the specificity for propionyl-CoA is ∼40-fold higher. This finding is qualitatively consistent with earlier studies on DEBS1 + TE, a bimodular derivative of DEBS (*25*, *27*) and

suggests that the loading AT domain is a significant contributor to the overall primer unit specificity of DEBS.

While the relaxed specificity of the loading AT provides diversity in the resulting polyketide, the extender AT domains have a strict specificity. We were unable to measure activity toward an unnatural substrate, setting the upper limit for the substrate specificity of unnatural substrates to be 3 orders of magnitude less than that of the natural substrates.

In an effort to rationally convert the loading AT of DEBS into a methylmalonyl transferase, Trp-222 was replaced by an Ala or Arg residue in two mutants. In the malonyl transferase from the *E. coli* fatty acid synthase, an Arg residue at the homologous position is thought to stabilize the carboxylate of the malonyl group (*28*). Replacement of the corresponding Arg residue in the mammalian fatty acid synthase led to a shift in specificity from a malonyl transferase to an acetyl transferase (*24*). However, the corresponding mutation in the loading AT domain of DEBS was insufficient to tilt its specificity toward methylmalonyl units, suggesting that multiple residues in the active site are likely to be important in stabilizing a carboxylate anion in the substrate. The complementary mutations were also introduced in the extender DEBS mmAT-6, in an effort to rationally convert this enzyme into a propionyl transferase. Again, a single mutation was insufficient to alter the specificity. Moreover, these mutations decreased the activities of the AT domains (by 10-fold in the loading AT domain and to the point where activity was undetectable for the wild-type substrate for the extender AT domains). These results highlight the subtle differences between acyl transferases from fatty acid synthases and polyketide synthases.

In summary, the development of a robust system for expression of acyl transferases as didomain proteins fused to a common ACP enabled us to generate substantial quantities of this pivotal component of modular PKSs for biochemical analysis. It also facilitated direct measurements of their specificity for different primer and extender units as well as alternative acceptor thiols. The availability of large quantities of these proteins may enable structural as well as presteady-state kinetic analysis of acyl transferases in the foreseeable future.

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