In Vitro Reconstitution and Analysis of the Chain Initiating Enzymes of the R1128 Polyketide Synthase†

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ABSTRACT: Biosynthesis of the carbon chain backbone of the R1128 substances is believed to involve the activity of a ketosynthase/chain length factor (ZhuB/ZhuA), an additional ketosynthase (ZhuH), an acyl transferase (ZhuC), and two acyl carrier proteins (ACPs; ZhuG and ZhuN). A subset of these proteins initiate chain synthesis via decarboxylative condensation between an acetyl-, propionyl-, isobutyryl-, or butyryl-CoA derived primer unit and a malonyl-CoA derived extender unit to yield an acetoacetyl-, β-ketopentanoyl-, 3-oxo-4-methylpentanoyl-, or β-ketohexanoyl-ACP product, respectively. To investigate the precise roles of ZhuH, ZhuC, ZhuG, and ZhuN, each protein was expressed in *Escherichia coli* and purified to homogeneity. Although earlier reports had proposed that ZhuC and its homologues played a role in *primer* unit selection, direct in vitro analysis of ZhuC showed that it was in fact a malonyl-CoA:ACP malonyltransferase (MAT). The enzyme could catalyze malonyl transfer but not acetyl- or propionyl-transfer onto R1128 ACPs or onto ACPs from other biosynthetic pathways, suggesting that ZhuC has broad substrate specificity with respect to the holo-ACP substrate but is specific for malonyl-CoA. Thus, ZhuC supplies *extender* units to both the initiating and elongating ketosynthases from this pathway. To interrogate the primer unit specificity of ZhuH, the kinetics of β-ketoacyl-ACP formation in the presence of various acyl-CoAs and malonyl-ZhuG were measured. Propionyl-CoA and isobutyryl-CoA were the two most preferred substrates of ZhuH, although acetyl-CoA and butyryl-CoA could also be accepted and elongated. This specificity is not only consistent with earlier reports demonstrating that R1128B and R1128C are the major products of the R1128 pathway in vivo, but is also in good agreement with the properties of the ZhuH substrate binding pocket, as deduced from a recently solved crystal structure of the enzyme. Finally, to investigate the molecular logic for the occurrence of not one but two ACP genes within the R1128 gene cluster, the inhibition of ZhuH-catalyzed formation of β-ketopentanoyl-ACP was quantified in the presence of apo-ZhuG or apo-ZhuN. Both apo-proteins were comparable inhibitors of the ZhuH catalyzed reaction, suggesting that the corresponding apo-proteins can be used interchangeably during chain initiation. Together, these results provide direct biochemical insights into the mechanism of chain initiation of an unusual bacterial aromatic PKS.

Bacterial aromatic polyketide synthases (PKSs),[1] also known as Type II PKSs, are analogous to Type II (bacterial and plant) fatty acid synthases in that they are composed of several monofunctional (and possibly bifunctional) proteins (*1*, *2*). They are primarily found in bacteria from the actinomyces group and are responsible for the biosynthesis of numerous clinically useful natural products including doxorubicin, mithramycin, and the tetracyclines. Two well-studied examples of bacterial aromatic PKSs are the actinorhodin (*act*) and the tetracenomycin (*tcm*) PKSs, which catalyze the assembly of an elongated poly-β-ketone chain from malonyl-CoA units (*3*, *4*). In both cases, the primer acetyl unit is derived from enzyme-catalyzed decarboxylation of a malonyl unit. Chain assembly requires four polypeptides, collectively referred to as the "minimal PKS" (Figure 1). Three of these—the ketosynthase (KS), the chain length factor (CLF, alternatively referred to as KS_β), and the acyl carrier protein (ACP)—are encoded within the polyketide biosynthetic gene cluster and are exclusively involved in biosynthesis of the natural product. The fourth protein, the malonyl-CoA:ACP acyltransferase (MAT), is shared between the fatty acid synthase and the aromatic PKSs in the bacterium.

Certain bacterial aromatic PKSs use non-acetate primers. For example, doxorubicin (*5*) and daunomycin (*6*) biosynthesis is primed by propionyl-CoA, whereas biosynthesis of the R1128 substances is variably primed by a variety of acyl-CoA species (*7*). The minimal PKSs (Figure 1) involved in these pathways must somehow suppress decarboxylative chain initiation of the KS/CLF heterodimer by malonyl-S-ACP in favor of incorporation of an alternative primer unit for chain elongation. Understanding the mechanistic basis for this phenomenon could provide a valuable route for

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[1] Abbreviations: ACP, acyl carrier protein; CoA, Coenzyme A; MAT, malonyl transferase; KS, ketosynthase; CLF, chain length factor; KR, ketoreductase; DH, dehydratase; ER, enoylreductase; PKS, polyketide synthase; LC/MS, liquid chromatography/mass spectroscopy; DTT, dithiothreitol; TCA, trichloroacetic acid; SDS-PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis.

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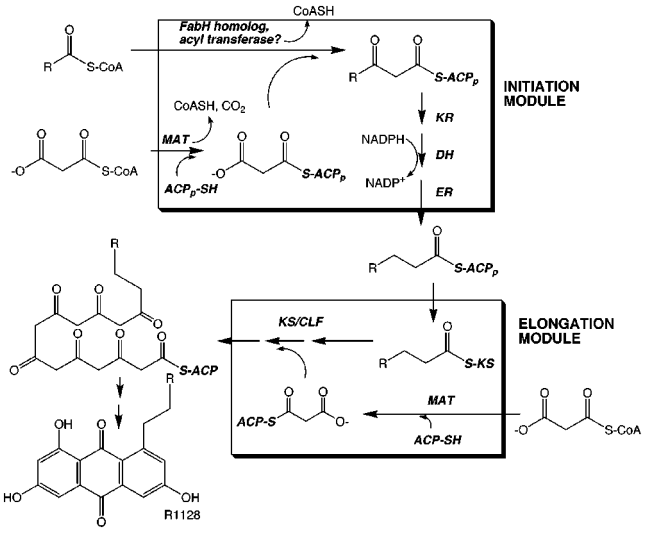

FIGURE 1: Chain Initiation and Elongation Modules of the R1128 PKS. Biosynthesis of the R1128 polyketide backbone requires the action of two "modules" of proteins (shown in italics, bold). The "initiation module" is comprised of a FabH-type ketosynthase, an acyl carrier protein (ACP_p), a malonyl-CoA:ACP_p acyltransferase (MAT), a ketoreductase (KR), a dehydratase (DH), an enoyl reductase (ER), and possibly yet another acyl transferase (ZhuC). Prior to this work, sequence analysis had revealed that ZhuH was the FabH-type ketosynthase, and either ZhuG or ZhuN were the ACP_p. No KR, DH, and ER candidates were encoded within the R1128 gene cluster. The "elongation module" is comprised of the KS/CLF heterodimer, a second ACP, and an MAT, which are also referred to as the minimal PKS. Sequence analysis had shown that the KS and CLF are encoded by *zhuA* and *zhuB*. The results reported here demonstrate that ZhuC can play the role of the initiating and elongating MAT, and that either ZhuG or ZhuN could play the role of the initiating ACP (ACP_p). Also demonstrated is the fact that the substrate specificity of ZhuH correlates with the observed relative abundance of the R1128 substances [R = CH_2CH_3 and R = $CH(CH_3)_2$ are the most abundant products whereas R = CH_3 and R = $CH_2CH_2CH_3$ are found in lower abundance]. The sources of KR, DH and ER activities remain unknown.

engineering novel functional groups into aromatic polyketides produced by Type II PKSs.

Cloning and sequence analysis of the doxorubicin (*5*), daunomycin (*6*), and R1128 (*7*) gene clusters provided initial insights into the mechanisms of chain initiation with non-acetate primers by the minimal PKS. In addition to minimal PKS genes, these gene clusters encode two or three other genes that are thought to be relevant to the chain initiation process: (i) a putative acyl transferase gene; (ii) a homologue of the *Escherichia coli fabH* gene (*8*), whose product catalyzes the first condensation between an acetyl primer and a malonyl-S-ACP extender during fatty acid biosynthesis; and (iii) in some cases, such as R1128 but not doxorubicin or daunomycin, a gene for a second ACP. In vivo studies with recombinant PKS gene clusters have demonstrated that the *fabH* homologue dictates, at least in part, the choice of a propionyl primer unit for the doxorubicin and daunomycin backbones (*9*, *10*). The role of the acyl transferase has been unclear, although it has been suggested that this enzyme plays a role in primer unit selection and/or incorporation (*7*, *9*). Likewise, the second ACP has also been proposed to play a role in primer unit selection and/or incorporation (*7*). Finally, in cases such as the R1128 substances, it appears that the primer unit is not only condensed to a malonyl extender unit, but the carbonyl of the resulting β-ketoacyl-ACP product is converted into a methylene functional group before the chain is passed on to the KS−CLF heterodimer (*7*). The ketoreductase (KR), dehydratase (DH), and enoyl reductase (ER) responsible for this reductive process are unknown, but as they are not encoded within the R1128 gene cluster, they may be shared with the host's fatty acid synthase (as is the case for the MAT). Thus, it appears that incorporation of a non-acetate primer into the product of a Type II PKS requires the activity of a "chain initiation module" comprised of a initiating ketosynthase, one or more acyl transferases, one or more ACPs and, in some cases, a KR, DH, and ER (Figure 1).

In an attempt to understand the mechanistic principles associated with initiating the biosynthesis of the R1128 polyketide backbone, we expressed, purified, and analyzed the two noncanonical enzymes encoded within this PKS gene cluster—the FabH homologue (ZhuH) and the acyl transferase (ZhuC). To facilitate these studies we also expressed, purified, and prepared different forms of the two ACPs encoded by the gene cluster (ZhuG and ZhuN). In conjunction with the recently reported crystal structure of ZhuH (*11*), the results described here provide insights into the enzymatic basis for broad substrate specificity of the R1128 synthase.

EXPERIMENTAL PROCEDURES

Materials. The *E. coli* strain BL21(DE3) and the pET-28 expression vectors were from Novagen. The oligonucleotides were purchased from Operon. General supplies for DNA and protein purification, including Ni^{2+}-NTA, were from Qiagen. Pharmacia manufactured the HiTrapQ anion-exhchange column. [1-^{14}C]Acetyl-CoA (50 mCi/mmol), [1-^{14}C]propionyl-CoA (54 mCi/mmol), [1-^{14}C]butyryl-CoA (4 mCi/mmol), and [2-^{14}C]malonyl-CoA (52 mCi/mmol), were obtained from Moravek Biochemicals. All other chemicals, including unlabeled Coenzyme A derivatives, were from Sigma and the highest available purity unless otherwise noted.

Construction of Expression Plasmids for ZhuC, ZhuH, ZhuN, and ZhuG. The gene encoding each of these proteins from the R1128 pathway was individually amplified by PCR using pHU235 as the template (*7*). In each case, oligonucleotide primers were used to introduce an *Nde*I restriction site overlapping the start codon and a *Hin*dIII site immediately following the stop codon. After the PCR product was ligated into the pCR-Blunt vector (Invitrogen) the integrity of the respective gene was confirmed by nucleotide sequencing (Stanford DNA sequencing facility). Following restriction digestion and ligation of the resulting *Nde*I/*Hin*dIII fragment into pET-28, the vector was transformed into *E. coli* BL21-(DE3).

General Expression and Purification Methods. E. coli transformants were grown using Luria-Bertani medium containing 50 mg/L of the appropriate antibiotic. The lysis buffer used to resuspend pelleted cultures contained 300 mM NaCl, 50 mM sodium phosphate, pH 8.0, and 10 mM imidazole.

Expression and Purification of ZhuH. Transformed *E. coli* were grown at 30 °C for 48 h. The cultures were pelleted and resuspended in lysis buffer. Tween-20 (1% v/v) was added and the cells were lysed by sonication. The soluble fraction was isolated by centrifugation and the supernatant was loaded onto a Ni^{2+}-NTA resin. The resin was washed

with 10 column volumes of lysis buffer containing 25 mM imidazole before the His_6-tagged protein was eluted using lysis buffer with 250 mM imidazole. The protein solution was diluted into 50 mM Tris, pH 8.0 (buffer A) and further purified by anion-exchange chromatography (2 mL/min) with a gradient of 20−60% buffer B (buffer A with 1 M NaCl). The His_6-ZhuH protein eluted at approximately 300 mM NaCl. The fractions containing ZhuH were concentrated then stored at −80 °C in 5 mM DTT, 5 mM HEPES (pH 7.5), 25 mM NaCl, and 10% (v/v glycerol) after buffer exchange.

Expression and Purification of ZhuC. Transformed *E. coli* were grown at 37 °C until $A_{600nm} = 0.7$ before the cultures were cooled to 30 °C and then induced with 0.1 mM isopropyl-β-D-galactopyranoside. The cultures were then grown for an additional 14 h. Cell lysis and Ni^{2+}-NTA affinity chromatography were carried out identically as described above for ZhuH. The eluate from the Ni^{2+}-NTA column was further purified by anion-exchange chromatography and ZhuC eluted as a single peak at 350 mM NaCl. The purified protein was buffer exchanged with 5 mM DTT, 5 mM HEPES (pH 7.5), and 25 mM NaCl before storing at −80 °C.

Expression and Purification of the Acyl Carrier Proteins from the R1128 PKS. Following growth of transformed *E. coli* at 37 °C until $A_{600nm} = 0.8$, the cultures were induced with 0.1 mM isopropyl-β-D-galactopyranoside. Cultures were then grown for an additional 20 h. Cell lysis and Ni^{2+}-NTA affinity chromatography were carried out identically as described above for ZhuH. The eluate from the Ni^{2+}-NTA column was loaded onto a Vydac C18 reversed-phase column, catalog no. 201TP510. Solvents A and B were composed of water and acetonitrile containing trifluoroacetic acid (0.1% v/v). The apo- and holo-ACPs were separated using a gradient of 45−60% over 30 min. The fractions were identified by electrospray mass spectrometry and refolded by buffer exchange into 50 mM Tris, pH 8.0, followed by overnight incubation at 4 °C. The ACPs were then loaded onto an anion-exchange column and eluted (2 mL/min) with a gradient of 0 to 100% 1 M NaCl over 30 min. The ACP proteins eluted at approximately 400 mM NaCl. Samples were exchanged into buffer containing 100 mM phosphate, pH 7.0, 2 mM DTT and stored at −80 °C.

Preparation of Malonyl-ZhuG. The broad-specificity phosphopantetheinyl transferase, Sfp (*12*, *13*), was purified and used as described previously (*14*). The phospohopantetheinyl transfer reaction contained apo-ZhuG (150 μM; purified as above), malonyl-CoA (600 μM), and Sfp (15 μM) in a solution of 10 mM $MgCl_2$, 2 mM EDTA, 2 mM DTT, and 100 mM phosphate, pH 6.6. The mixture was incubated at 37 °C and the reaction was monitored by analytical HPLC. Once complete conversion of apo- to malonyl-ACP was detected, the reaction mixture was purified by anion-exchange chromatography by first washing with 100 mM NaCl to remove Sfp and then eluting the malonyl-ZhuG (2 mL/min) with a gradient of 0 to 100% 1 M NaCl over 30 min. Malonyl-ZhuG eluted with a similar retention volume to apo- or holo-ZhuG at a concentration of 400 mM NaCl. The sample was exchanged into buffer containing 10 mM phophate, pH 6.6, and stored at −80 °C. Conversion of apo-ZhuG into malonyl-ZhuG was confirmed by LC/MS.

Identification and Detection of Biosynthetic Intermediates. Reaction volumes were 10 μL and were buffered with 100 mM sodium phosphate, pH 7.0, and 1 mM DTT. To visualize acyl-ZhuH, 1.0 μg of enzyme was incubated with 100 μM radiolabeled acyl-CoA at 30 °C for 5 min. For production of acyl-ACPs, 2.0 μg of holo-ACP was incubated at 30 °C for 15 min with radiolabeled malonyl-, acetyl-, or propionyl-CoA in the presence of 0.1 μg of ZhuC. The reaction mixtures were then separated by SDS−PAGE (4 to 20% gradient) and the gels were analyzed by autoradiography.

Substrate Specificity Analysis of Chain Initiation Mechanism. For commercially available radiolabeled CoA derivatives (acetyl, propionyl, and butyryl), a trichloroacetic acid (TCA) precipitation method was utilized to detect the acetoacetyl-, β-ketopentanoyl-, or β-ketohexanoyl-ACP products, respectively. Reaction volumes were 25 μL and contained 100 mM sodium phosphate, pH 7.0, and 1 mM DTT. To quantify the dependence of ZhuH on malonyl-ZhuG and propionyl-CoA, the concentrations of these two species were varied between 0 and 30 and 0−50 μM, respectively, to generate a 4 × 4 plot. To analyze the acyl-CoA substrate specificity of ZhuH, steady-state kinetics were measured in the presence of variable acyl-CoA substrates (0−200 μM) and a fixed concentration of malonyl-ZhuG (25 μM). In each case the CoA derivative and malonyl-ZhuG were combined and incubated for 15 min at 30 °C. Reactions were initiated by adding 10 ng of enzyme, and time points were taken during the linear stage of the reaction. The β-ketoacyl-ACP product was precipitated by quenching the reaction with 1 mL of 10% TCA at 4 °C. Bovine serum albumin (20 μL of a 10 mg/mL solution) was added and the solution was vortexed for 15 s before centrifuging at 16000*g* for 5 min. The supernatant was removed and the pellet was washed with 0.5 mL of cold TCA. The sample was vortexed for 2 s, and the insoluble fraction was again isolated by centrifugation and removal of the supernatant. The pelleted protein, containing the β-ketoacyl-ACP product, was dissolved in 1.0 mL of pH 8.0, 100 mM phosphate containing 2% sodium dodecyl sulfate. The vial was then washed with an additional 0.5 mL of water, and the two fractions containing the radiolabeled protein were combined. Supernatants and pellets were mixed with 4 mL of scintillation fluid and analyzed on a liquid scintillation counter.

Substrate Specificity Assay for Nonradiolabeled Substrates. Reaction conditions were similar to those described for the TCA assays. In 25 μL of total reaction volume, 25 μM malonyl-ZhuG, 100 μM acetyl-CoA, and 100 μM competing CoA (propionyl, isobutyryl, or isovaleryl) were mixed. The reaction mixture was buffered with 100 mM sodium phosphate, pH 7.0, and 1 mM DTT. Reactions were incubated at 30 °C for 30 min, and the relative amounts of each β-ketoacyl-ACP product was determined by LC/MS. Simultaneous detection was possible for the *m*/*z* peaks at 1668.2 (acetyl), 1670.2 (propionyl), 1672.2 (isobutyryl), and 1674.2 (isovaleryl) atomic mass units. All of these peaks correspond to a +7 charged β-ketoacyl-ACP species. Similar results were also obtained by examining other integral protein ionization states, although +7 was the most intense *m*/*z* peak in all cases. The ratio of the integrated peaks for the products of interest was used to quantify the enzyme selectivity.

RESULTS

Expression and Purification of R1128 Proteins. The chain initiating ketosynthase (ZhuH) and acyl transferase (ZhuC)

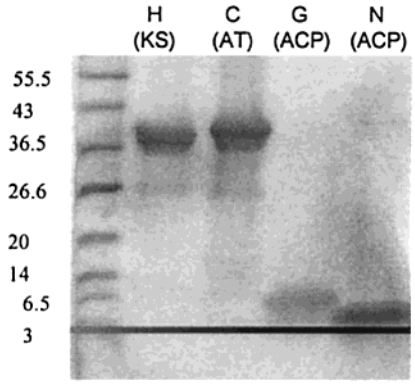

FIGURE 2: SDS−PAGE gel of purified proteins. Lanes from left to right are molecular weight standards, ZhuH, ZhuC, ZhuG, and ZhuN.

were overexpressed in *E. coli* by construction of individual pET-28a derivatives containing the desired genes in frame with the N-terminal His_6 tag from the vector. The desired proteins were purified from cell lysates using Ni^{2+}-affinity and anion-exchange chromatography (Figure 2). The typical yields of highly purified ZhuH and ZhuC were >1 mg/L.

The R1128 gene cluster includes two ACP genes, each of which was cloned into the pET-28a expression vector. The resulting proteins, ZhuG and ZhuN, were purified in a manner similar to that described above for ZhuH and ZhuC. Following Ni^{2+}-affinity and anion-exchange chromatography, the samples of ZhuG and ZhuN appeared >95% pure by SDS−PAGE (Figure 2). The purified samples of ZhuG and ZhuN were further analyzed by reversed-phase HPLC and mass spectrometry. Both protein preparations were found to contain two major fractions, the holo and apo forms (with and without the phosphopantetheine prosthetic group, respectively). However, the relative ratios of the holo and apo forms were dramatically different. Whereas ZhuG was expressed primarily as the apo form (apo:holo = 80:20), ZhuN was predominantly obtained in the holo form (apo:holo < 20:80). Repeated attempts to produce apo-ZhuN resulted in consistently poor yields of this form of the ACP.

To accurately measure the kinetics of the ZhuH-catalyzed reaction, a suitable malonyl-ACP was needed in high purity. The presence of both apo- and holo-ACP in the FPLC-purified fractions of ZhuG and ZhuN presented a problem. Methods for converting either apo- or holo-ACP into malonyl-ACP are known (*15*, *16*), but quantitative conversion of a mixed pool of apo- and holo-ACP into malonyl-ACP is difficult. To overcome this problem, we developed a preparative HPLC method for separating the two forms of ZhuG and ZhuN. Through the optimization of a standard reversed-phase HPLC gradient method on a C-18 semi-preparative column (see Experimental Procedures), we were able to fully resolve the two major products in each case (Figure 3). Although the HPLC-purified materials were indistinguishable by SDS−PAGE, their identify could be clearly confirmed by mass spectrometry (Table 1). The product in each case was found to be lacking the N-terminal methionine. Multi-milligram quantities of apo-ZhuG could thus be prepared; however, the yield of purified apo-ZhuN was low.

The ACP proteins from the HPLC purification presumably elute in a denatured state due to the presence of >30%

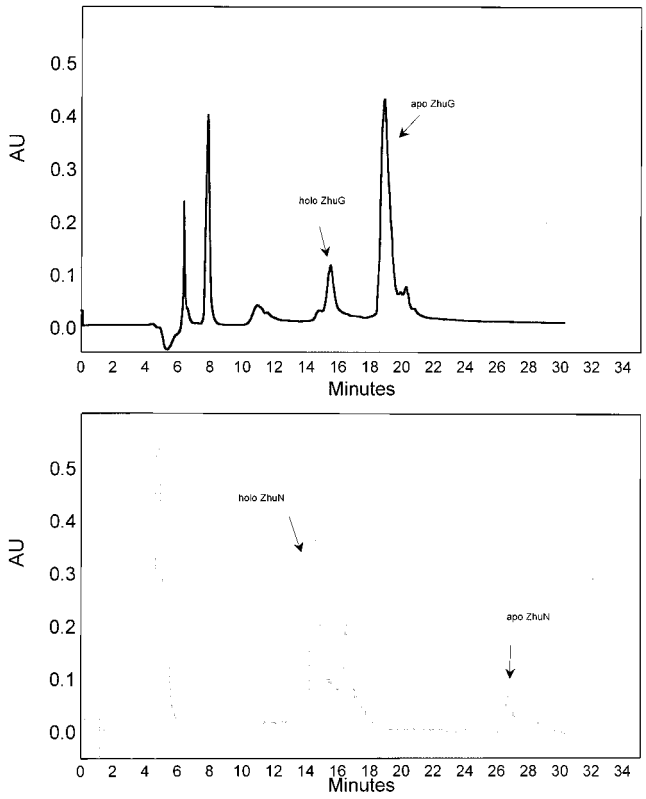

FIGURE 3: Separation of holo and apo ACPs. (Top) Preparative HPLC trace from the purification of apo- and holo-ZhuG from the FPLC purified mixture. (Bottom) Preparative HPLC trace from the purification of apo- and holo-ZhuN from the FPLC-purified mixture. The holo and apo fractions are labeled in each trace.

Table 1: Mass Spectral Data for Acyl Carrier Proteins

ACP species	expected mass (Da)[a]	observed mass (Da)
apo-ZhuG	11 245	11 247
holo-ZhuG	11 584	11 587
malonyl-ZhuG	11 670	11 673
apo-ZhuN	10 677	10 677
holo-ZhuN	11 016	11 018

[a] The calculated masses include removal of N-terminal Met.

acetonitrile. To restore their native structure, fractions containing ACP proteins were refolded by solvent exchange into aqueous buffer followed by overnight incubation at 4 °C. The refolded samples were subjected to anion-exchange chromatography to obtain a sharp peak with a retention time comparable to a ZhuG or ZhuN sample that had not been subjected to the denaturation-renaturation sequence. (The refolded apo-and holo-ACPs eluted at a salt concentration of 450 mM NaCl.) Perhaps more importantly the holo-ACP fractions were labeled efficiently with malonyl-CoA in an MAT-catalyzed reaction, and the apo-ACP fractions served as substrates for Sfp (see below).

Preparation of Malonyl-ZhuG. Given the substantially superior yields of apo-ZhuG as compared to apo-ZhuN, ZhuG was selected for the synthesis of malonyl-ACP. By using the broad-specificity phosphopanteheinyl transferase from *Bacillus subtilis*, Sfp (*12*, *13*), we were able to achieve complete conversion of apo-ZhuG into malonyl-ZhuG in vitro. The reaction was reproducibly performed on a multi-milligram scale and yielded protein, which after a rapid purification step using anion-exchange chromatography,

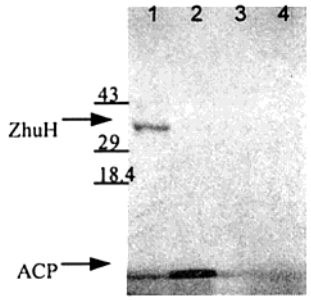

FIGURE 4: Autoradiograph of gel showing covalent intermediates. Lane 1, incubation of ZhuH with [^{14}C]propionyl-CoA in the presence of holo-ZhuG. Lane 2, incubation of holo-ZhuG and ZhuC with [^{14}C]malonyl-CoA. Lane 3, incubation of holo-ZhuG and ZhuC with [^{14}C]propionyl CoA, Lane 4, incubation of holo-ZhuG and ZhuC with [^{14}C]acetyl-CoA. The labeled malonyl-ACP band can be seen at the bottom of the gel in lane 3; this band is absent in the other lanes. The unresolved darkening at the bottom of the gel below the ACP is presumed to be unreacted [^{14}C]acyl-CoA, since its migration is faster than any of the protein components in the reaction mixture and it comigrates with [^{14}C]acyl-CoA loaded on the gel in the absence of any protein. Incubation of the holo-ACP with [^{14}C]-acyl-CoA in the absence of ZhuC resulted in no radiolabeling of the ACP (data not shown).

could be used as a substrate in the ZhuH-catalyzed reaction (see below). Its purity and identity was confirmed by HPLC/MS (Table 1).

Identification of Biosynthetic Intermediates. Once ZhuH, ZhuC, ZhuG, and ZhuN were available in a purified form, they could be assayed for their ability to produce synthetic intermediates relevant to the putative R1128 chain initiation mechanism (Figure 1). Specifically, we wished to attempt detection of various acyl-ZhuH, acyl-ZhuG, and acyl-ZhuN adducts. Incubation of ZhuH with radiolabeled acetyl-CoA, propionyl-CoA, or butyryl-CoA, followed by SDS−PAGE-autoradiographic analysis, revealed that the protein could be labeled in each case. (For example, see Figure 4, lane 1 for the experiment involving propionyl-CoA.) As precedented by earlier studies on a ZhuH homologue from *M. tuberculosis*, direct detection of the β-ketoacyl-ACP via SDS−PAGE-autoradiography was not possible, presumably due to the instability of this species (*17*). The putative acyltransferase, ZhuC, was assayed for its ability to produce acyl-ACP in the presence of radiolabeled acyl-CoA. Incubation of ZhuC with holo-ACP (either ZhuG or ZhuN; only data for ZhuG is shown in Figure 4) in the presence of malonyl-CoA (lane 2), acetyl-CoA (lane 3), or propionyl-CoA (lane 4) provided insights into its substrate specificity. Malonyl-CoA strongly labeled the holo-ACP, whereas acetyl- or propionyl-CoA did not. The discrimination by ZhuC between these radiolabeled substrates is noteworthy because the concentration of malonyl-CoA was 17 μM in this assay, whereas the acetyl- or propionyl-CoA reactions contained 100 μM substrate of comparable specific activity. Incubation of ZhuC and radiolabeled malonyl-CoA with a number of holo-ACPs from other aromatic polyketide biosynthetic pathways, including actinorhodin, frenolicin, granaticin, and doxorubicin ACPs, also yielded the corresponding radiolabeled ACPs (data not shown.). Thus, it can be concluded that ZhuC is not a primer unit transferase. Rather, it is a malonyl-CoA:ACP acyltransferase (MAT) that can catalyze malonyl transfer onto virtually any ACP from a Type II PKS.

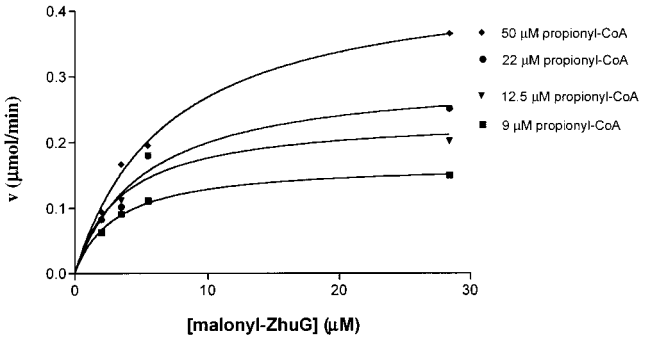

FIGURE 5: Kinetic analysis. Rate of the ZhuH reaction vs [ACP] vs [propionyl-CoA].

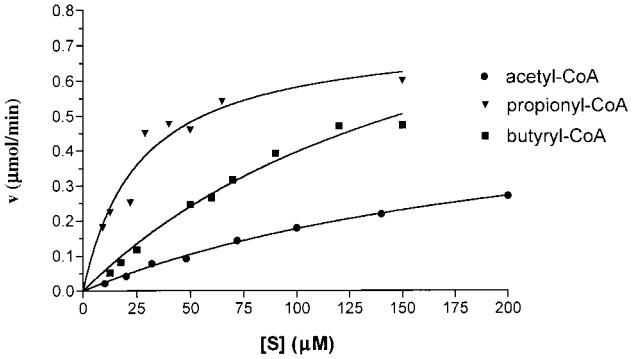

FIGURE 6: Substrate specificity of ZhuH. Rate of the ZhuH reaction vs [acetyl-CoA] or [propionyl-CoA] or [butyryl-CoA]. The malonyl-ACP concentration was kept constant at 25 μM.

Substrate Specificity of ZhuH. As shown in Figure 1, the condensation reaction catalyzed by ZhuH requires two substrates: (1) an acyl-CoA, which supplies the primer unit, and (2) a malonyl-ACP, which serves as a source of the nucleophilic extender unit. We wished to analyze the substrate specificity of ZhuH with respect to both of these substrates. Toward this end several steady-state kinetic experiments were performed. As shown in Figure 5, a 4 $\times$ 4 analysis of the dependence of the ZhuH reaction on malonyl-ZhuG and propionyl-CoA concentrations yielded a k_{cat} of 60 min^{-1}, a $K_{M,ZhuG}$ of 7 μM, and a $K_{M,Pr-CoA}$ of 30 μM. To compare the specificity of ZhuH for acetyl-CoA, propionyl-CoA, and butyryl-CoA (each of which is available in a radiolabeled form), more extensive v versus [S] plots were generated at a fixed malonyl-ZhuG concentration of 25 μM (Figure 6). The results of these experiments are summarized in Table 2, and show that propionyl-CoA is a significantly superior substrate to both acetyl-CoA and butyryl-CoA. Finally, the relative preference of ZhuH for ZhuG versus ZhuN was indirectly evaluated by comparing the ability of either apo-ACP to inhibit the ZhuH-catalyzed reaction involving malonyl-ZhuG. (A more direct evaluation of this property was not possible due to the extremely low quantities of available apo-ZhuN, which precluded preparation of reagent quantities of pure malonyl-ZhuN; see above.) As seen in Figure 7, apo-ZhuG is a competitive inhibitor of ZhuH-catalyzed formation of β-ketopentanoyl-ZhuG (K_I = 40 μM). In a side-by-side comparison of HPLC-purified and renatured apo-ZhuG and apo-ZhuN, both proteins were found to be comparable inhibitors of the ZhuH-catalyzed reaction (Table 3), suggesting that either ACP could participate in the initiation of the R1128 polyketide backbone.

Table 2: Kinetic Data for ZhuH Catalyzed Condensation of Malonyl-ZhuG with Various Alkyl-CoA Thioesters

	source of acyl primer unit (R−CoA)				
kinetic parameter	acetyl	propionyl	isobutyryl	butyryl	isovaleryl
k_{cat} (min^{-1})	58	74	nd[a]	113	nd
K_m (μM)	230	26	nd	184	nd
k_{cat}/K_m	0.25	2.8	nd	0.61	nd
$(k_{cat}/K_m)r/(k_{cat}/K_m)$acetyl	1	11.2	nd	2.4	nd
k_{rel}[b]	1	3.8	3.9	nd	0.1

[a] nd = not determined. [b] Product ratio in competition assay analyzed by LC/MS (see text for details).

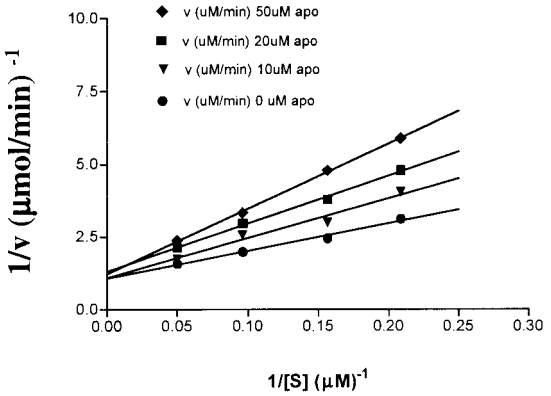

FIGURE 7: Inhibition of the ZhuH reaction by apo-ZhuG. The propionyl-CoA concentration was kept constant at 50 μM.

Table 3: Comparison of Inhibition of the ZhuH-Catalyzed Reaction by Apo-ZhuN and Apo-ZhuG

[apo-ACP]	relative activity[a]
0 μM	100%
10 μM apo-ZhuG	71%
50 μM apo-ZhuG	28%
10 μM apo-ZhuN	66%
50 μM apo-ZhuN	45%

[a] As measured by TCA-precipitated dpm after a 5 min incubation of 20 μM malonyl-ZhuG, 54 μM propionyl-CoA, and varying concentrations of the apo-ACP. A 5 min time-point was chosen for the quench based on the results of kinetic analysis shown in Figure 7.

Competition Assays for Nonradiolabeled CoA Derivatives. Due to the inavailability of other radiolabeled acyl-CoA substrates, a full set of steady-state kinetic parameters could only be obtained for acetyl-, propionyl-, and butyryl-CoA. However, the selectivity of ZhuH for unlabeled substrates, including isobutyryl- and isovaleryl-CoA was estimated by LC/MS analysis. Reaction conditions were essentially identical as for the radiolabeled substrates except that two competing acyl-CoA substrates were co-incubated with ZhuH and malonyl-ZhuG. Acetyl-CoA was used as the reference, and was present in all reactions along with an equal concentration of propionyl-, isobutyryl-, or isovaleryl-CoA. The reactions were allowed to proceed to ~80% completion and analyzed by LC/MS. Although baseline chromatographic separation could not be achieved for individual β-ketoacyl-ACP products in the same sample, the use of selective mass spectrometric detection at the desired mass range for each product enabled quantitation of the relative amounts of each product (see Experimental Procedures). These results, which are summarized in Table 2, suggest that isobutyryl-CoA is a comparable substrate to propionyl-CoA, whereas isovaleryl-CoA is a much poorer substrate than either acetyl-CoA or butyryl-CoA.

DISCUSSION

The recently characterized R1128 biosynthetic gene cluster (*7*) was found to include three genes—*zhuC*, *zhuG* (or alternatively *zhuN*), and *zhuH*—not found in typical bacterial aromatic PKS gene clusters. Since the polyketide backbones of R1128 substances are derived from non-acetate primer units, it was proposed that these gene products participate in the alternative chain initiation process. Although sequence analysis provided some insights into the function of these proteins, understanding their precise role in R1128 biosynthesis required the biochemical analysis reported here. Fortunately, each of the proteins under consideration could be readily expressed in *E. coli* and purified from crude lysates (Figure 2).

The dramatic difference between the ratios of apo- to holo-ACP produced in the cases of ZhuG and ZhuN is noteworthy. Previous studies demonstrated that heterologous expression of *Streptomyces* ACPs in *E. coli* can result in a wide range of posttranslational phosphopantetheinylation in vivo (*15*), presumably due to a combination of variable expression levels and affinity for the endogenous holo-ACP synthetase enzyme (*16*). For example, the *tcm* ACP was expressed nearly 100% in the apo form, whereas 30% of the *gra* ACP expressed in *E. coli* was phosphopantetheinylated in vivo (*15*). Since apo- and malonyl-ACP were the two forms of ACP of greatest relevance for this study, recombinant ZhuG was more useful than recombinant ZhuN.

Since homologues of *zhuC* are only found in bacterial aromatic PKS gene clusters where non-acetate primers are used, it was assumed that ZhuC and its homologues play a role in chain initation. However, our studies on ZhuC have revealed that it is an *extender* unit acyl transferase, not a *primer* unit acyl transferase. As such it is functionally equivalent to the *Streptomyces coelicolor* MAT that catalyzes malonyl transfer onto both the fatty acid and Type II PKS ACPs found in the organism (*3*). Why then are dedicated genes for the MAT found in some PKS gene clusters but not others? The answer to this question might emerge from more detailed mechanistic analysis of ZhuC and/or its homologues.

Perhaps most significantly, the present study has analyzed the substrate specificity of the ZhuH protein from the R1128 pathway. Its relatively broad substrate specificity correlates well with the observed distribution of R1128 substances in vivo. In vitro ZhuH has greatest specificity toward propionyl and isobutyryl primer units; the corresponding products, R1128B and R1128C, are the most abundant in the natural host (*18*) and in heterologous hosts (*7*). Also tolerated are acetyl and butyryl primer units, as evidenced by direct biochemical studies described here and by the isolation of R1128A and R1128D in vivo (*18*). Thus, ZhuH appears to

be the primary gatekeeper for primer units in R1128 biosynthesis. Not only is this consistent with earlier reports on doxorubicin and daunomycin biosynthesis (*9*, *10*), but it also provides the biosynthetic engineer with a particularly useful PKS subunit for the generation of novel aromatic polyketide products. For example, to the extent that ZhuH is able to accept and elongate alternative primer units (e.g., haloacetyl, halopropionyl, aryl, and possibly aminoacyl primers), it may be possible to produce R1128 analogues with medicinally useful functionality. Moreover, given the recent description of the acyl chain-binding pocket of ZhuH at high resolution (*11*), ZhuH is an attractive target for protein engineering with the goal of systematically enlarging its binding pocket to accept bulkier primer units.

Finally our results also shed some light on the presence of not one but two ACP genes in the R1128 gene cluster. Sequence analysis had suggested that ZhuG might be a component of the chain initiation module, whereas ZhuN would be a component of the elongating module (Figure 1) (*7*). However, our results described here suggest that both ZhuG and ZhuN are interchangeable substrates for ZhuC and ZhuH. Therefore, either these ACPs are truly interchangeable or discrimination between the two proteins lies at the level of the KS−CLF or the yet-to-be-discovered KR, DH, and ER which participate in the chain initiation module of the R1128 synthase.

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