Ketosynthases in the Initiation and Elongation Modules of Aromatic Polyketide Synthases Have Orthogonal Acyl Carrier Protein Specificity†

Yi Tang,‡ Taek Soon Lee,§ Seiji Kobayashi,‡ and Chaitan Khosla*,‡,§,||

Departments of Chemical Engineering, Chemistry, and Biochemistry, Stanford University, Stanford, California 94305-5025

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ABSTRACT: Many bacterial aromatic polyketides are synthesized by type II polyketide synthases (PKSs) which minimally consist of a ketosynthase-chain length factor (KS-CLF) heterodimer, an acyl carrier protein (ACP), and a malonyl-CoA:ACP transacylase (MAT). This minimal PKS initiates polyketide biosynthesis by decarboxylation of malonyl-ACP, which is catalyzed by the KS-CLF complex and leads to incorporation of an acetate starter unit. In non-acetate-primed PKSs, such as the frenolicin (*fren*) PKS and the R1128 PKS, decarboxylative priming is suppressed in favor of chain initiation with alternative acyl groups. Elucidation of these unusual priming pathways could lead to the engineered biosynthesis of polyketides containing novel starter units. Unique to some non-acetate-primed PKSs is a second catalytic module comprised of a dedicated homodimeric KS, an additional ACP, and a MAT. This initiation module is responsible for starter-unit selection and catalysis of the first chain elongation step. To elucidate the protein−protein recognition features of this dissociated multimodular PKS system, we expressed and purified two priming and two elongation KSs, a set of six ACPs from diverse sources, and a MAT. In the presence of the MAT, each ACP was labeled with malonyl-CoA rapidly. In the presence of a KS-CLF and MAT, all ACPs from minimal PKSs supported polyketide synthesis at comparable rates (k_{cat} between 0.17 and 0.37 min^{-1}), whereas PKS activity was attenuated by at least 50-fold in the presence of an ACP from an initiation module. In contrast, the opposite specificity pattern was observed with priming KSs: while ACPs from initiation modules were good substrates, ACPs from minimal PKSs were significantly poorer substrates. Our results show that KS-CLF and KSIII recognize orthogonal sets of ACPs, and the additional ACP is indispensable for the incorporation of non-acetate primer units. Sequence alignments of the two classes of ACPs identified a tyrosine residue that is unique to priming ACPs. Site-directed mutagenesis of this amino acid in the initiation and elongation module ACPs of the R1128 PKS confirmed the importance of this residue in modulating interactions between KSs and ACPs. Our study provides new biochemical insights into unusual chain initiation mechanisms of bacterial aromatic PKSs.

Type II polyketide synthases (*1*) (PKSs)[1] are responsible for the biosynthesis of aromatic polyketides, many of which are pharmacologically valuable compounds (Figure 1). The organization of genes within PKSs and functions of encoded enzymes parallel closely those of type II fatty acid synthases (FASs) (*2*). Heterologous expression of proteins from different type II PKS clusters in engineered hosts (*3*) has allowed the elucidation of individual protein functions and has facilitated the rational reassembly of enzymes toward generation of novel polyketides (*4*, *5*). The works by our laboratory (*6*−*12*) and others (*13*, *14*) have revealed a "minimal PKS" that is essential for the construction of a polyketide chain (Figure 2A). The minimal PKS is a set of four monofunctional enzymes that catalyze the initiation and elongation of a polyketide chain from malonyl-CoA. The ketosynthase (KS) and the chain length factor (CLF) form a heterodimer (KS-CLF, also termed $KS_{\alpha}KS_{\beta}$) that catalyzes condensation reactions between successive malonyl units. The KS-CLF also controls the overall chain length (*3*) and influences the regiospecificity (*6*) of some of the cyclization events that occur on the full-length chain. An acyl carrier protein (ACP) shuttles malonyl units to the active site of the KS-CLF in the form of a malonyl-ACP. Acyl transfer between malonyl-CoA and ACP is catalyzed by the malonyl-CoA:ACP transacylase (MAT), which is shared between FAS and PKSs (*15*).

In well-characterized PKSs such as those involved in the biosynthesis of actinorhodin (**1**, *act*) (*12*) and tetracenomycin (**3**, *tcm*) (*16*), the KS-CLF is primed by an acetate unit through decarboxylation of a malonyl-ACP. This acetyl group is then transferred to the active site of KS, followed by dissociation of ACP and association of a second equivalent of malonyl-ACP. Residues that are critical for the decarboxylation reaction have been located on both the KS (*17*) and CLF (*18*) subunits, although the exact priming mechanism remains elusive. Some aromatic PKSs use non-

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* To whom correspondence should be addressed. E-mail: ck@chemeng.stanford.edu.

‡ Department of Chemical Engineering.

§ Department of Chemistry.

|| Department of Biochemistry.

[1] Abbreviations: PKS, polyketide synthase; FAS, fatty acid synthase; CoA, coenzyme A; KS, ketosynthase; CLF, chain length factor; ACP, acyl carrier protein; MAT, malonyl-CoA:ACP transacylase; KSIII, ketoacyl synthase III; TCA, trichloroacetic acid.

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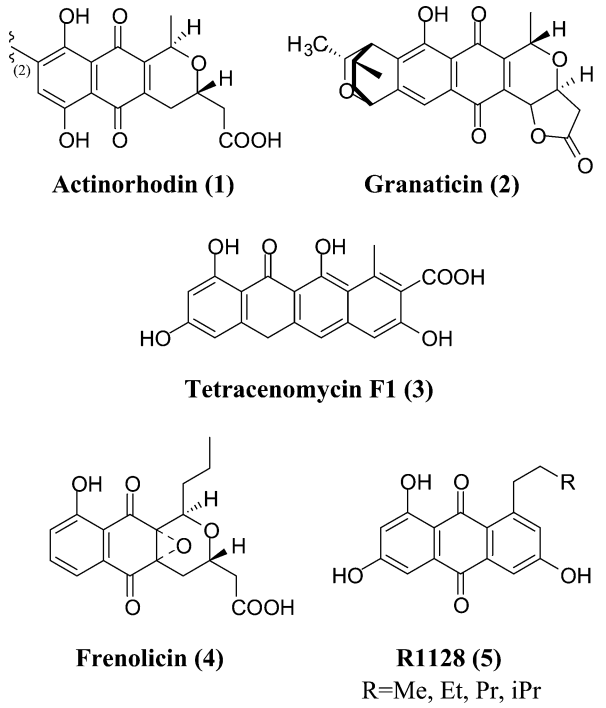

FIGURE 1: Aromatic polyketides. Actinorhodin, granaticin, and tetracenomycin are primed with acetate primers. Frenolicin is primed with a butyryl group, and R1128 is primed with a variety of acyl groups as shown.

acetate primers (*19*). For example, frenolicin (*20*), an antimalarial agent produced by *Streptomyces roseofulvus*, is primed by a butyryl unit [although nanaomycin (*21*), the acetate-primed analogue of frenolicin, is also produced by the same PKS in significantly higher abundance]; R1128, an estrogen receptor antagonist (*22*−*24*), is primed by a variety of primer units, excluding acetate; and daunorubicin, a widely used antitumor drug, is primed by a propionyl unit (*25*−*27*). Two unique proteins have been located in the gene clusters of these PKSs, and are believed to be responsible for chain initiation (*28*): (1) a ketosynthase (FrenI in *fren* PKS, ZhuH in R1128 PKS, and DpsC in *dnr* PKS) homologous to the FAS ketoacyl synthase III (KSIII) and (2) a second ACP (FrenJ in *fren* PKS and ZhuG in R1128 PKS) in addition to the putative minimal PKS ACP (FrenN and ZhuN). DpsG is the only ACP found in the *dnr* PKS (*25*). The KSIII from a bacterial FAS is responsible for chain initiation, and catalyzes condensation between malonyl-ACP and a short chain acyl-CoA to yield a β-ketoacyl-ACP (*29*, *30*). The β-carbonyl of this intermediate is then reduced before the nascent fatty acid chain is transferred to another KS that catalyzes further chain elongation. It has been shown recently that the FAS KSIII from *Streptomyces glaucescens* demonstrates activity toward both its endogenous FAS ACP and *Escherichia coli* FAS ACP, but not toward its endogenous PKS ACP TcmM (*31*). Thus, ZhuH and FrenI may play an essential and similar role in the R1128 and frenolicin pathways, respectively (Figure 2B) (*31*). The recently elucidated X-ray crystal structure of ZhuH has provided a useful starting point for mechanistic dissection of chain initiation (*32*).

The role of the additional ACP found in the *fren* and R1128 clusters is not understood. We have previously shown that both apo-ZhuG and apo-ZhuN inhibited the ZhuH-catalyzed condensation between propionyl-CoA and malonyl-ZhuG, suggesting equivalent roles for both proteins in the priming mechanism (*33*). Furthermore, only one ACP is found in the PKS cluster of daunorubicin, thus raising the possibility of ACP gene redundancy in *fren* and R1128. Deciphering the precise choices of ACPs by the loading and elongation modules is critical in uncovering the unusual chain initiation mechanism of non-acetate priming PKSs. Toward this end, we expressed and purified two KS-CLFs, two KSIII proteins, and six ACPs from a variety of PKSs. Two in vitro assays were used to directly probe the abilities of the ACPs to support either polyketide synthesis catalyzed by the KS-CLF or chain initiation catalyzed by KSIII. From kinetic analysis of KS-CLF−ACP and KSIII−ACP interactions, we show that FrenJ and ZhuG are indispensable in the priming of *fren* and R1128 PKSs, respectively, whereas FrenN and ZhuN are the preferred components of the elongation module.

EXPERIMENTAL PROCEDURES

Materials. [1-^{14}C]Propionyl-CoA (55 mCi/mmol) and [1-^{14}C]acetyl-CoA (50 mCi/mmol) were purchased from Moravek Biochemicals. [2-^{14}C]Malonyl-CoA (55 mCi/mmol) was from American Radiolabeled Chemicals. Flag peptide, Anti-FLAG M2 monoclonal antibody, Anti-FLAG M1 agarose affinity gel, and all other biochemicals, including unlabeled CoA derivatives, were purchased from Sigma. Phenyl-Sepharose resin and Hitrap Q anion-exchange column were purchased from Amersham Biosciences.

DNA Manipulation and Mutagenesis. All cloning steps were performed in *E. coli* strain XL-1 Blue. Site-directed mutagenesis was performed using the Quickchange kit from Stratagene. Primers CTCGCTCGCGCTG*TAC*GAGACCGCCGCC and CGACTCGCTCGCCGTC*CTG*GAGGTCGTCAC and their complementary oligonucleotides were used to introduce the M42Y mutation into ZhuN and the Y45L mutation into ZhuG.

Protein Expression and Purification. (1) act and tcm KS-CLF. *Streptomyces coelicolor* strains CH999/pSEK38 (*17*) and CH999/pSEK33 (*10*) were used to obtain *act* KS-CLF and *tcm* KS-CLF, respectively. pSEK38 encodes the *act* CLF with an N-terminal FLAG tag. Spores suspensions of each strain were used to inoculate 3 × 1 L of SMM medium containing 50 mg/L thiostrepton. Mycelia from the stationary phase cultures (3 days) were collected by centrifugation and resuspended in 40 mL of disruption buffer. Mycelia were disrupted with a French press, and insoluble cellular debris was removed by centrifugation (24000*g* for 1 h). DNA was precipitated by adding 0.2% polyethyleneimine (PEI) and was removed by centrifugation (24000*g* for 1 h). KS-CLF proteins were precipitated between 30 and 50% $(NH_4)_2SO_4$. Precipitated proteins were redissolved in buffer A [100 mM NaH_2PO_4, 2 mM DTT, and 2 mM EDTA (pH 7.4)] and loaded onto a phenyl-Sepharose column preequilibrated with buffer B [buffer A with 1.5 M $(NH_4)_2SO_4$]. The following gradient was applied to the column: 100% buffer B from 0 to 30 min, 40% buffer B from 30 to 60 min, 10% buffer B from 60 to 200 min, and 0% buffer B from 200 to 240 min. Both *act* and *tcm* KS-CLF complexes eluted near the end of the gradient. Fractions containing *act* KS-CLF were pooled and buffer exchanged into 50 mL of TBS buffer [50 mM Tris (pH 7.4), 0.15 M NaCl, and 10 mM $CaCl_2$] and loaded onto a column packed with Anti-FLAG M1 agarose affinity

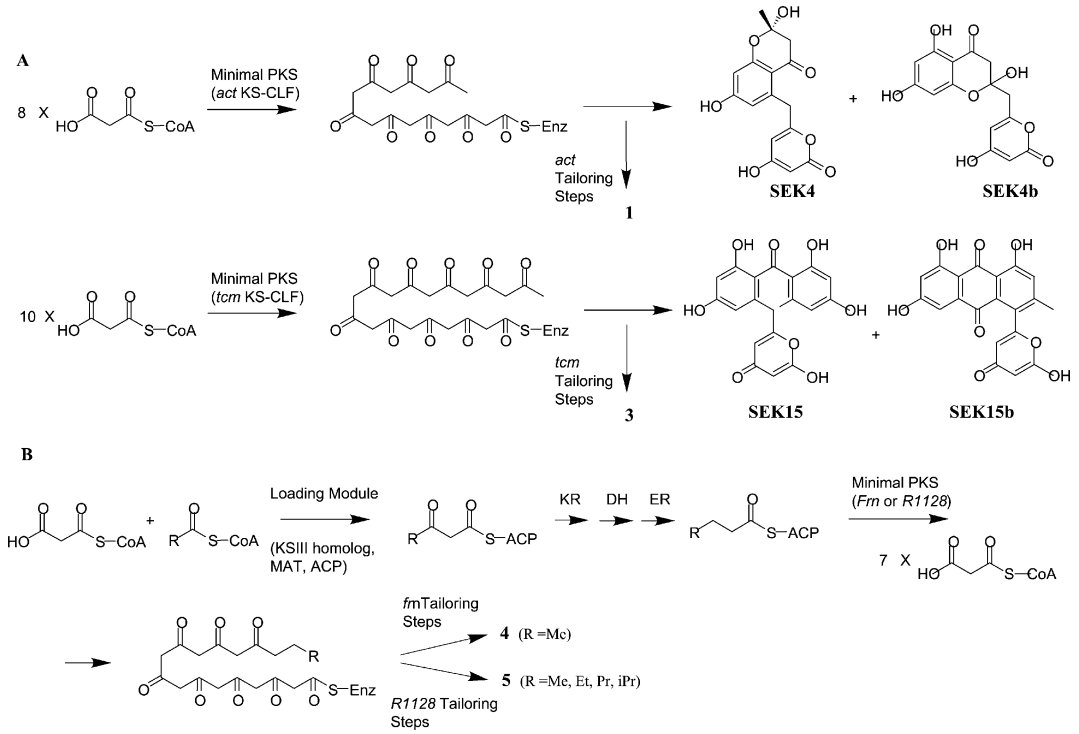

FIGURE 2: Steps in the synthesis of polyketides by minimal PKSs and proposed mechanism of chain initiation in *fren* and R1128 aromatic PKSs. (A) Minimal PKSs that consist of *act* or *tcm* KS-CLF are able to synthesize C16 or C20 polyketides from eight or ten malonyl-CoAs, respectively. The minimal PKS initiates polyketide synthesis through decarboxylation of one malonyl-ACP. Additional malonyl-ACPs are recruited by and condensed with KS-CLF to elongate the polyketide chain until the desired chain length is reached. Without additional tailoring enzymes, SEK4/4B and SEK15/15B are formed by *act* and *tcm* minimal PKS, respectively. (B) From *fren* and R1128 PKSs, a loading module is proposed to synthesize the starter unit. The loading module consists of a KSIII homologue, an additional ACP, and MAT. KSIII catalyzes the condensation between ACP and acyl-CoA to form the β-ketoacyl-ACP. The β-ketoacyl-ACP is then reduced to an acyl-ACP by most likely homologues of FAS KR, DH, and ER. The minimal PKS is primed acyl-ACP, followed by full-length polyketide synthesis as in panel A.

gel (5 mL). The column was washed with 30 mL of TBS, and KS-CLF was eluted with 3 × 5 mL of TBS containing 100 μg/mL FLAG peptide. The eluent was concentrated, buffer exchanged into buffer A containing 20% glycerol, aliquoted, flash-frozen with liquid nitrogen, and stored at −80 °C.

Fractions containing *tcm* KS-CLF were pooled and buffer exchanged into buffer A and loaded onto a Hitrap Q column preequilibrated with buffer A. The following gradient was applied to the column: 0% buffer C (buffer A with 400 mM NaCl) from 0 to 20 min, 35% buffer C from 20 to 30 min, 35% buffer C from 30 to 40 min, 60% buffer C from 40 to 70 min, and 100% buffer C from 70 to 100 min. The target protein eluted at 50% buffer C. The eluent was concentrated, aliquoted, and frozen as described above.

(2) KSIII Purification. The gene encoding FrenI was amplified from plasmid pIJ5214 (*20*) and cloned into pET28a to yield pYT30. *E. coli* strains BL21(DE3)/pESM8 and BL21(DE3)/pYT30 were used to obtain ZhuH and FrenI, respectively. Purification procedures for ZhuH were described in detail previously (*33*). FrenI was purified using a similar protocol.

(3) ACP and MAT Purification. *S. coelicolor* MAT was expressed in *E. coli* and purified as described previously (*15*). *E. coli* expression strains BAP1/pFRN, BAP1/pSK73, BAP1/pANS401, BAP1/pESM10, BAP1/pESM11, and BAP1/pYT21 were used to obtain holo versions of FrenN, Gra ACP (*34*), DpsG, ZhuG, ZhuN, and FrenJ, respectively. Protein expression was induced at an OD_{600} of 0.5 with 100 μM IPTG and allowed to proceed at 30 °C for 10 h. The cells were then harvested and lysed with sonication. ZhuG, ZhuN, and FrenJ contained N-terminal six-His tags and were purified using Ni−NTA resin under native conditions (Qiagen), followed by anion-exchange chromatography on a HiTrap Q column (from 0 to 100% NaCl in 60 min, ACPs elute between 300 and 400 mM NaCl). FrenN, Gra ACP, and DpsG were first partially purified by anion-exchange chromatography, and then purified to homogeneity using a phenyl-Sepharose chromatography step [from 1.5 to 0 M $(NH_4)_2SO_4$ in 100 min, ACPs elute between 300 and 100 mM $(NH_4)_2SO_4$]. The relative amounts of the holo and apo forms of each ACP were quantified by MALDI-TOF mass spectrometry. FrenN, Gra ACP, DpsG, and ZhuN were all found to be nearly 100% in the holoprotein form. ZhuG and FrenJ were found to be only 40% in the holoprotein form under the same expression conditions. The phosphopantetheinyl transferase, Sfp, was used to convert the partially apo forms of the ACP to holo-ACP. The phosphopantetheinyl transfer reaction was performed as described previously in vitro and was monitored by HPLC (*35*). CoASH and Sfp were removed with anion-exchange chromatography upon completion of the reaction. Holo-ACP proteins were buffer exchanged into buffer A containing 20% glycerol, flash-frozen, and stored at −80 °C. All protein concentrations were determined by the Bradford method with the Bio-Rad protein kit.

MAT Labeling Assay. The conversion of holo-ACP to malonyl-ACP catalyzed by MAT was assayed in vitro

using [^{14}C]malonyl-CoA. Reactions were performed in reaction buffer [100 mM NaH_2PO_4 (pH 7.0) and 1 mM DTT] at 25 °C. Each reaction mixture contained 200 μM [2-^{14}C]malonyl-CoA (55 mCi/mmol) and 100 μM holo-ACP in a final volume of 35 μL. Reactions were initiated by adding 1 nM (final concentration) MAT. Aliquots (10 μL) were removed at 30, 60, and 120 s, and reactions were quenched by adding 3× SDS−PAGE sample buffer. The quenched fractions were applied to a 4 to 20% SDS gel, and ACPs were separated from MAT and malonyl-CoA by electrophoresis. [^{14}C]Malonyl-labeled ACPs were visualized and quantified by using a phosphoimager (InstantImager 2024, Packard).

KS-CLF Titration Assay. This assay detects the amount of polyketides synthesized by the minimal PKS from radiolabeled malonyl-CoA. The product is either SEK4/4b for *act* KS-CLF or SEK15/15b for *tcm* KS-CLF. Assays were performed at 30 °C in 100 μL of reaction buffer containing 10% glycerol. Holo-ACP concentrations were between 2 and 100 μM. All reaction mixtures contained 100 nM MAT and 1.5 mM malonyl-CoA. For reactions involving FrenN, ZhuN, Gra ACP, and DpsG, the specific activities of malonyl-CoA were 1 mCi/mmol and the concentration of the KS-CLF heterodimer was 0.7 μM. To facilitate detection of products in reaction mixtures containing poor substrates such as ZhuG and FrenJ, the specific activities of CoA used were increased to 4.5 mCi/mmol and the concentrations of KS-CLF were doubled. The reaction was initiated by adding malonyl-CoA. Six aliquots (15 μL) were taken within 40 min, and each was added to 12 μL of quench buffer (12.5% SDS). Each quenched mixture was vortexed and extracted with 300 μL of ethyl acetate twice. The combined organic phases were evaporated to dryness and redissolved in 20 μL of ethyl acetate. The reaction products were separated by thin-layer chromatography (TLC) and quantified with a phosphoimager as described previously (*12*).

KSIII Assay. This assay detects the condensation between radiolabeled acyl-CoA and malonyl-ACP catalyzed by either ZhuH or FrenI. Malonyl-ACP is generated in situ from holo-ACP, malonyl-CoA, and MAT. Reactions were performed in a final volume of 40 μL at 25 °C. Each reaction mixture contained 100 μM [^{14}C]acyl-CoA, 300 μM malonyl-CoA, and 50 μM holo-ACP. In reaction mixtures containing ZhuH, [1-^{14}C]propionyl-CoA (11 mCi/mmol) was used as the acyl donor, while [1-^{14}C]acetyl-CoA (10 mCi/mmol) was used in assays containing FrenI. Reaction mixtures were pre-incubated together with 100 nM MAT for 5 min. Condensation was initiated by adding 0.05 μM (final concentration) ZhuH or FrenI. Reactions in aliquots (6 μL) taken within 30 min were quenched with 1 mL of 10% cold trichloroacetic acid (TCA), which precipitates the β-ketoacyl-ACP. The solution was incubated on ice for 10 min after the addition of 20 μL of BSA (10 mg/mL), followed by centrifugation (14000*g* for 5 min). The pellet was washed with 0.5 mL of 10% cold TCA and dissolved in 0.5 mL of 98% formic acid. Each sample tube was washed with an additional 0.5 mL of water; the two fractions were combined with 4 mL of scintillation fluid, and the amount of radioactivity was determined with a liquid scintillation counter (Beckman LS3801).

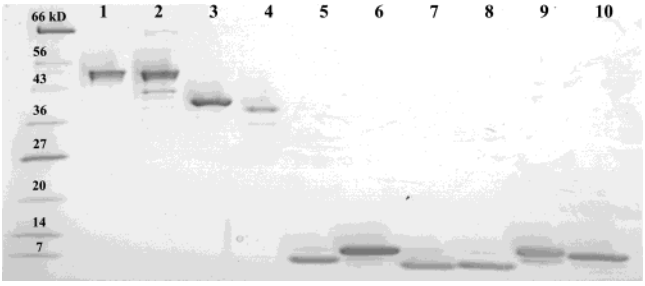

FIGURE 3: SDS−PAGE of purified proteins assayed in this work: lane 1, *act* KS-CLF; lane 2, *tcm* KS-CLF; lane 3, ZhuH; lane 4, FrenI; lane 5, FrenN; lane 6, ZhuN; lane 7, Gra; lane 8, DpsG; lane 9, ZhuG; and lane 10, FrenJ.

Table 1: Proteins Studied in This Work

acyl carrier protein	
FrenN	frenolicin minimal PKS
FrenJ	frenolicin PKS initiation module
ZhuN	R1128 minimal PKS
ZhuG	R1128 PKS initiation module
Gra-ORF3	granaticin minimal PKS
DpsG	daunorubicin minimal PKS
ketosynthase	
actI ORF1-ORF2	actinorhodin KS-CLF
tcmKL	tetracenomycin KS-CLF
ZhuH	R1128 KSIII
FrenI	frenolicin KSIII
MAT	malonyl-CoA:ACP acyltransferase

RESULTS

Protein Expression and Purification. To assess the protein−protein interactions between ACPs and ketosynthases from different PKS clusters, we employed two types of assays. The PKS activity assay reconstitutes the minimal PKS in vitro and monitors the kinetics of full-length polyketide synthesis (*11*). The KSIII-dependent assay determines the initial rates of the acyl transfer reaction between acyl-CoA and malonyl-ACP (*33*). We purified two KS-CLF heterodimers, two KSIII homodimers, and six different ACPs for use in these assays (Figure 3). KS-CLF proteins from the actinorhodin and tetracenomycin clusters were expressed in *S. coelicolor* CH999 containing plasmids pSEK38 and pSEK33, respectively. A FLAG tag was fused to the N-terminus of *act* CLF, which facilitated its purification along with stoichiometric amounts of *act* KS by using an anti-FLAG affinity column. The high specificity of the column resulted in recovery of the heterodimeric complex (>95% pure). The *tcm* KS-CLF was purified in two steps as described previously (*36*) to >80% purity as judged by SDS−PAGE. Typical yields of purified KS-CLF proteins were between 0.5 and 1 mg/L of culture volume.

The KSIII homologues FrenI and ZhuH were expressed in *E. coli* as N-terminal hexa-His fusion proteins. Purification, activity (*33*), and structural (*32*) studies of ZhuH have been previously described. FrenI was purified using a similar protocol to >90% homogeneity.

The six ACPs studied in this work are listed in Table 1. Among the six, FrenJ and ZhuG have been proposed to participate in the priming of *fren* and R1128 PKSs, respectively. Each ACP was expressed in the engineered *E. coli* host BAP1 (*37*), which encodes the phosphopantetheinyl transferase, *sfp*, on its chromosome. ZhuN, ZhuG, and FrenJ contained six-His affinity tags and were each batch purified

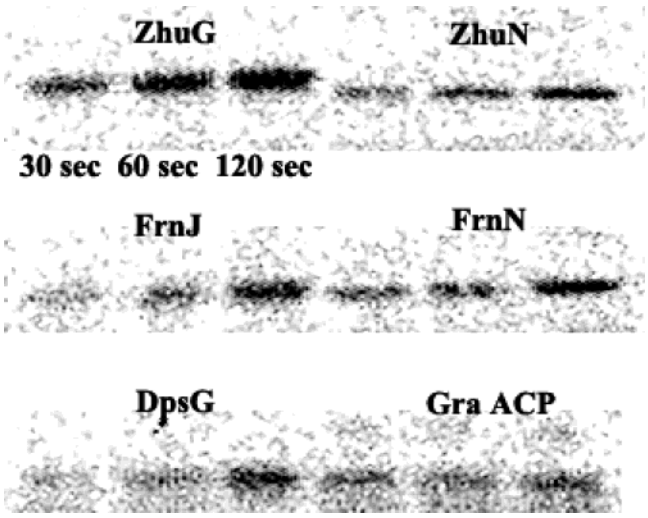

FIGURE 4: MAT-catalyzed labeling of holo-ACP by malonyl-CoA. The extents of labeling of ACP by [2-^{14}C]malonyl-CoA at three time points (30, 60, and 120 s) are visualized by SDS−PAGE and autoradiography. In each reaction mixture, 1 nM MAT, 100 μM holo-ACP, and 200 μM malonyl-CoA are present. All ACPs are labeled with MAT at comparable rates. The k_{cat} values for ZhuG, ZhuN, FrenJ, FrenN, DpsG, and Gra ACP are 8330, 3617, 6518, 5016, 5839, and 3340 min^{-1}, respectively.

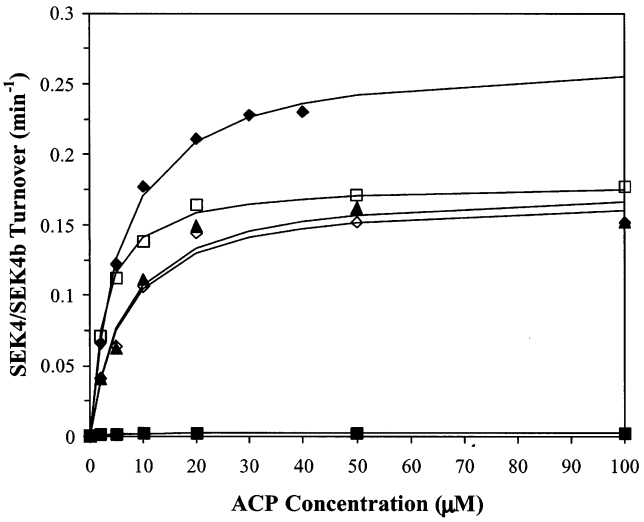

FIGURE 5: Representative kinetics of the PKS activity assay. The kinetics of SEK4/SEK4B synthesis starting from malonyl-CoA (1.5 mM), catalyzed by *act* KS-CLF (0.7 μM), MAT (100 nM), and different ACPs, are shown. Minimal PKS ACPs FrenN (◆), ZhuN (□), DpsG (◇), and Gra ACP (▲) all supported polyketide formation at comparable rates. Priming ACPs ZhuG (■) were not active in the assay. Not shown is FrenJ. For details, see the text and Table 2.

using Ni−NTA resin, followed by an anion-exchange chromatography step. FrenN, Gra ACP, and DpsG were expressed in native forms and were purified using two chromatography steps. All ACPs were purified to >95% homogeneity. Holo-ACPs are required for the in vitro assays described in this report. The relative amounts of the holo and apo forms of the proteins were determined by MALDI-TOF mass spectroscopy. All ACPs except ZhuG and FrenJ were in nearly 100% holo forms when expressed in BAP1. Apo-ZhuG and apo-FrenJ (~60%) were converted to the corresponding holo forms in vitro by using the purified Sfp protein (*35*). Holo-ZhuG and holo-FrenJ were further purified using anion-exchange chromatography upon completion of the reaction.

MAT Malonyl Transfer Assay. Both the PKS and KSIII assays require in situ generation of malonyl-ACP, which can be accomplished by the addition of malonyl-CoA and catalytic amounts of MAT. To deconvolute the protein−protein interactions between ACP and ketosynthases from potentially interfering MAT−ACP interactions, we must first evaluate the kinetics of malonyl transfer for different MAT−ACP pairs. Toward this end, we determined the initial rates of the malonylation reaction by SDS−PAGE and autoradiography. As shown in Figure 4, at 1 nM MAT, we were able to observe a linear increase in the intensity of the labeled ACP band within the first 2 min for all ACPs. In the absence of MAT, no ACP was labeled (data not shown). All six ACPs were labeled to a comparable extent within the assay time period. The k_{cat} difference between the best substrate (ZhuG, $k_{cat} \sim 8330$ min^{-1}) and the least preferred substrate (Gra ACP, $k_{cat} \sim 3340$ min^{-1}) is less than 3-fold. The rate of PKS ACP malonylation by MAT is slower than that of the endogenous FAS ACP (450 k_{cat} s^{-1}) (*38*). Under PKS and KSIII assay conditions where the MAT concentrations are 100 nM and the rates of product formation for both KS-CLF- and KSIII-catalyzed reactions are significantly lower than that of MAT (see below), we do not expect the rates of malonyl-ACP formation to affect the apparent KS−ACP interactions.

PKS Activity Assay. The abilities of the ACPs to support polyketide synthesis were assessed using an assay that included reconstituted *act* or *tcm* KS-CLF. The expected products of *act* and *tcm* minimal PKSs are SEK4/4B and SEK15/15B, respectively (*10*). The levels of polyketide accumulation were monitored by radio-TLC and were used to derive the kinetic parameters of ACP−KS-CLF interactions. Under conditions that include a high malonyl-CoA concentration, a linear increase in the extent of polyketide accumulation was observed within 40 min. The results of titrating different holo-ACPs into the *act* KS-CLF-catalyzed reaction are shown in Figure 5. Consistent with in vivo studies (*3*, *39*), ACPs derived from all minimal PKSs (FrenN, ZhuN, Gra ACP, and DpsG) supported polyketide synthesis effectively. The k_{cat} values based on combined SEK4 and SEK4B levels ranged between 0.17 and 0.27 min^{-1}. Similar results were observed for titrations of the same set of ACPs into the mixture for the *tcm* KS-CLF-catalyzed reaction (Table 2). These results suggest that ACPs derived from minimal PKSs can be interchanged without significant kinetic penalties, regardless of the source of the ACP.

In contrast to the interchangeable nature of ACPs derived from minimal PKSs, ACPs derived from initiation PKS modules (FrenJ and ZhuG) are poor substrates of KS-CLF heterodimers (Figure 5). In the presence of the *act* KS-CLF, the rates of SEK4/SEK4B synthesis supported by either malonyl-FrenJ or malonyl-ZhuG were attenuated by at least 50-fold compared to that of FrenN. The k_{cat}/K_m values listed in Table 2 represent upper bound estimates, since quantification of the low-intensity TLC spots was hampered by background counts. The *tcm* KS-CLF was more tolerant of ACPs from initiation modules, although here too ACPs from minimal PKSs are clearly preferred.

Three of the six ACPs that have been investigated contain N-terminal hexa-His sequences (ZhuG, ZhuN, and FrenJ).

Table 2: Activities of *act* and *tcm* KS-CLF toward Different ACPs[a]

	FrenN	ZhuN	Gra ACP	DpsG	FrenJ	ZhuG
act KS-CLF[b]						
K_m (μM)	5.8 ± 0.40	2.7 ± 0.18	6.4 ± 1.5	6.5 ± 1.2	ND[c]	ND
k_{cat} (min^{-1})	0.27 ± 0.005	0.18 ± 0.002	0.17 ± 0.011	0.18 ± 0.008	ND	ND
k_{cat}/K_m (min^{-1} mM^{-1})	47	66	27	27	1	1
k_{rel}[d]	1.0	1.4	0.57	0.58	0.02	0.02
tcm KS-CLF[e]						
K_m (μM)	2.3 ± 0.34	1.41 ± 0.22	1.6 ± 0.32	2.6 ± 0.77	17.3 ± 3.3	ND
k_{cat} (min^{-1})	0.32 ± 0.010	0.31 ± 0.008	0.36 ± 0.012	0.37 ± 0.022	0.30 ± 0.018	ND
k_{cat}/K_m (min^{-1} mM^{-1})	139	219	225	142	17.3	5.2
k_{rel}	1.0	1.6	1.6	1.02	0.12	0.04

[a] Procedures of KS-CLF titration assays are given in Experimental Procedures. [b] For *act* KS-CLF minimal PKS, the polyketides quantified in each assay are SEK4 and SEK4B (Figure 2). [c] Given the low activities of these substrates, individual K_m and k_{cat} values cannot be accurately determined. The k_{cat}/K_m values given for FrenJ and ZhuG represent upper bound estimates. [d] k_{rel} is defined as the ratio of the k_{cat}/K_m value relative to that of FrenN. [e] For *tcm* KS-CLF minimal PKS, the polyketides quantified in each assay are SEK15 and SEK15B (Figure 2).

To confirm that KS-CLF−ACP interactions were unaffected by these histidine tags, we performed *act* minimal PKS activity assays with ZhuG and ZhuN after treating the two ACPs with thrombin. Under these conditions, the histidine tags were completely cleaved after thrombin digestion, as indicated by MALDI-TOF mass spectrometry (data not shown). The titration results were identical to those observed with His-tagged variants of ZhuG and ZhuN (data not shown).

KSIII Activity Assay. The recognition of different ACPs by KSIII homologues FrenI and ZhuH can be assessed using an acyl transfer assay. Meadows et al. have shown that ZhuH catalyzes chain elongation between acetyl-, propionyl-, isobutyryl-, or butyryl-CoA and malonyl-ZhuG, with propionyl-CoA being the best substrate (*33*). The formation of radiolabeled β-ketopentanoyl-ACP was followed using a TCA precipitation assay. Figure 6A shows the time course of ACP labeling in the presence of ZhuH and propionyl-CoA. ZhuG is the best substrate with an apparent k_{cat} value of 69 min^{-1}, consistent with the previous report. ZhuH exhibited high selectivity of the ACP substrate: the apparent k_{cat} value of ZhuG is nearly 10-fold higher than that of the next best substrate, FrenJ. Among the minimal PKS ACPs, only Gra ACP exhibited detectable labeling. FrenN, ZhuN, and DpsG were completely unlabeled.

To compare the ACP specificities of KSIII homologues, we performed the same assay with FrenI. We first determined the substrate specificity of FrenI toward acetyl-, propionyl-, and butyryl-CoA. We expected acetyl-CoA to be the preferred acyl donor group, since a butyryl group is found at C16 of frenolicin (Figure 1). We reasoned that the butyryl group arises from the sequential reduction of acetoacetyl-FrenJ by homologues of FabG, FabA, and FabI, in a fashion identical to that of the R1128 starter units. Our findings concurred with the hypothesis: the apparent k_{cat} for acetyl-CoA in this assay was 32.8 min^{-1} (Figure 6B), approximately 3-fold higher than that of propionyl-CoA, and 30-fold higher than that of butyryl-CoA (data not shown). We therefore used acetyl-CoA as the acyl donor in the FrenI−ACP assay, and the results are shown in Figure 6B. FrenI displayed less selectivity toward priming ACPs, and the apparent k_{cat} of ZhuG (17.6 min^{-1}) is reduced by less than 2-fold compared to that of FrenJ. Consistent with the results from the ZhuH−ACP titration assay, Gra ACP was the only minimal PKS ACP that was labeled with FrenI.

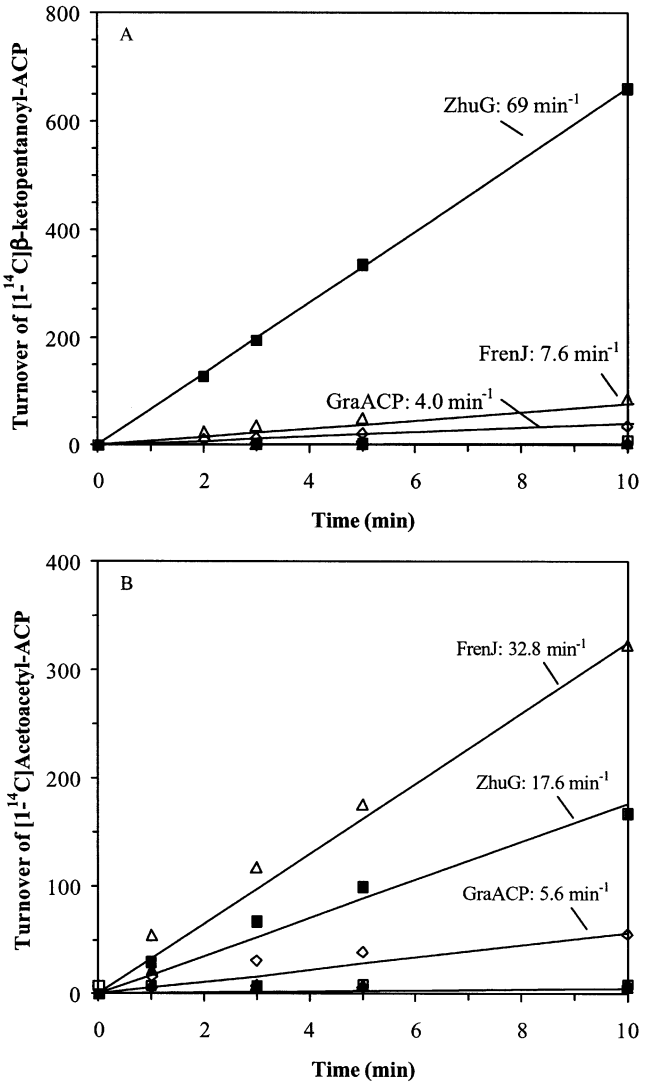

FIGURE 6: KSIII acyl transfer assay. (A) ZhuH (0.05 μM)-catalyzed transfer of [^{14}C]propionyl-CoA (100 μM) to malonyl-ACP. (B) FrenI (0.05 μM)-catalyzed transfer of [^{14}C]acetyl-CoA (100 μM) to malonyl-ACP. Malonyl-ACP was generated in situ from MAT (100 nM), malonyl-CoA (300 μM), and holo-ACP (50 μM). Priming ACPs ZhuG (■) and FrenJ (△) were competent acceptors of acyl groups in each assay. Minimal PKS ACPs FrenN (◆), ZhuN (□), and DpsG (◇) were not labeled by either acyl-CoA. Gra ACP (▲) showed low levels of labeling in both assays.

Sequence Alignment and ACP Mutagenesis. Sequence alignments of ACPs from different PKS clusters and FASs

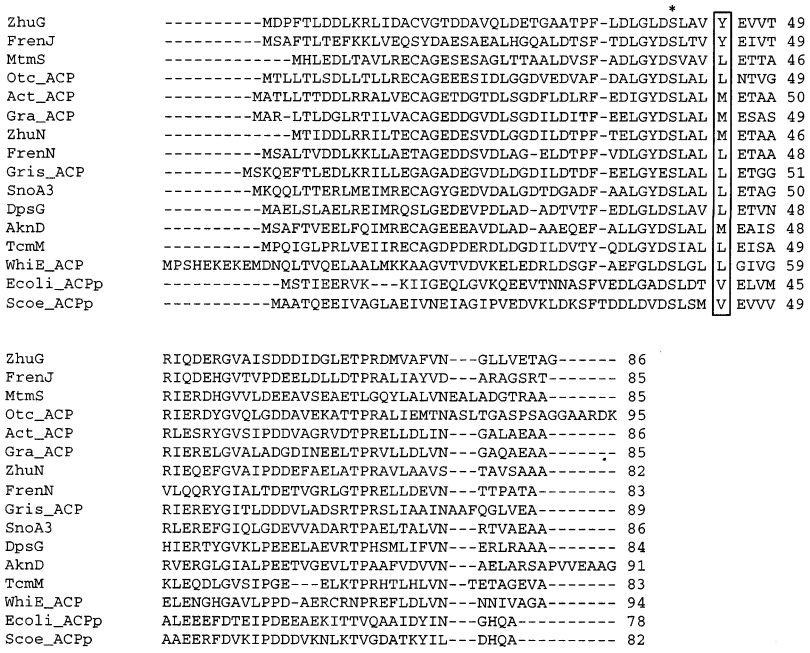

FIGURE 7: Multiple-sequence alignment of ACPs from different PKSs and FASs. The conserved catalytic serine residue is marked with an asterisk. The residue identified as being important in KSIII−ACP interactions is boxed. The PKS ACPs shown here but not mentioned in the text are as follows: MtmS (mithramycin), Otc_ACP (oxytetracycline), Act_ACP (actinorhodin), Gris_ACP (griseusin), SnoA3 (noglamycin), AknD (alkavinone), TcmM (tetracenomycin), and WhiE_ACP (*Streptomyces avermitilis* spore pigment). All these PKSs are primed with acetate primers. Ecoli_ACPp and Scoe_ACPp are the FAS ACP_p from *E. coli* and *S. coelicolor*, respectively.

Table 3: Activities of ZhuG, ZhuN, and Mutant ACPs in the ZhuH-Catalyzed Acyl Transfer Assay[a]

ACP	initial velocity (min^{-1})[b]	ACP	initial velocity (min^{-1})[b]
ZhuG	69	ZhuN	no activity
ZhuG-Y45L	42	ZhuN-M42Y	14.7

[a] ^{14}C-labeled propionyl-CoA was used as the acyl donor. [b] Initial velocities were calculated from time points taken in the first 10 min of the assay.

are shown in Figure 7. No major primary sequence differences are apparent among ZhuG, FrenJ, and the rest of the PKS ACPs. We note subtle sequence features unique only to the pair of priming ACPs. Helix II of ACPs (spanning approximately residues 40−50) has previously been noted to interact extensively with ketosynthases (*40*−*42*). The universally conserved serine (Ser41 in ZhuG) that carries the phosphopantetheinyl prosthetic arm is located within this helix. Residue 45 in both ZhuG and FrenJ is a tyrosine, whereas leucine (the consensus amino acid) or methionine is usually found at the corresponding position in all known minimal PKS ACPs. In the ACP from either the *E. coli* or *S. coelicolor* fatty acid synthase, this residue is a valine. Introduction of the bulky tyrosine residue into helix II may lead to a significantly altered secondary structure that facilitates recognition by PKS KSIII exclusively. To assess the importance of this residue, we performed site-directed mutagenesis on both ZhuG and ZhuN. The mutants ZhuG-Y45L and ZhuN-M42Y were expressed, purified, and phosphopantetheinylated as described for wild-type ACPs. In the PKS assay, the activity of neither mutant significantly changed when compared to that of wild-type ACP. However, the mutations affected the activities of the ACPs in the ZhuH-catalyzed condensation assay as shown in Table 3. Replacement of tyrosine with leucine in ZhuG resulted in a <2-fold decrease in the initial velocity. More notably, introduction of tyrosine within helix II of ZhuN allowed the ACP to be acylated at a rate that was 25% of that of ZhuG. The ZhuH−ACP assay demonstrates that while this residue is important in modulating KSIII−ACP interactions, its identity is not essential to facilitation of KS-CLF−ACP recognition.

DISCUSSION

Frenolicin and R1128 PKSs are the only two aromatic PKSs sequenced to date that contain both a KSIII homologue and an additional ACP. Our results have provided new insight into the roles of these additional ACPs. Specifically, we have shown that (1) secondary ACPs are indispensable for the activities of priming modules but (2) they are not appropriate substrates for KS-CLF heterodimers. Similarly, while ACPs from minimal PKSs are fully interchangeable among each other, they are unable to serve as substrates for either ZhuH or FrenI (with the exception of Gra ACP, which supports low levels of chain initiation). Thus, it appears that the initiation and elongation modules of PKSs such as the frenolicin and R1128 PKSs have orthogonal ACP specificity. It is noteworthy that DpsG from the daunorubicin pathway is a good substrate for KS-CLF proteins, but not for KSIII proteins. Since the daunorubicin gene cluster encodes only a single ACP, it may be that DpsC (the KSIII homologue from that pathway) has ACP recognition features that resemble those of KS-CLF heterodimers.

Our previous work with ZhuG, ZhuN, and ZhuH led to the suggestion that ZhuG and ZhuN are interchangeable substrates of ZhuH (*33*). The proposal was based on the observation that apo-ZhuN, when added to a reaction mixture containing ZhuH, malonyl-ZhuG, and propionyl-CoA, was able to competitively inhibit chain initiation. In this study, we have directly evaluated the ability of ZhuH to interact with malonyl-ZhuN, and shown thereby that malonyl-ZhuN is not a substrate of ZhuH.

The concomitant production of nanaomycin and frenolicin by the *fren* PKS in *S. roseofulvus* presumably arises as a result of competition between decarboxylative priming of the KS-CLF by malonyl-FrenN and chain transfer from butyryl-FrenJ to the KS-CLF. In contrast, the R1128 PKS does not produce polyketides derived via decarboxylative priming. Thus, the R1128 KS-CLF must accept acyl chains from acyl-ZhuG in preference to catalyzing decarboxylation of malonyl-ZhuG or malonyl-ZhuN. Acyl-ZhuG must be a significantly better substrate for KS-CLF than malonyl-ZhuG to initiate polyketide synthesis, highlighting the importance of the ACP prosthetic group in modulating KS−ACP interactions. Reconstitution of the R1128 KS-CLF with both the loading module and minimal PKS components will provide insight into how decarboxylative priming is suppressed in R1128 PKS. In turn, this insight may lead to the engineering of novel starter units into aromatic polyketides.

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