Enzymes Involved in Fatty Acid and Polyketide Biosynthesis in *Streptomyces glaucescens*: Role of FabH and FabD and Their Acyl Carrier Protein Specificity†

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ABSTRACT: Malonyl acyl carrier protein (ACP) is used as an extender unit in each of the elongation steps catalyzed by the type II dissociated fatty acid synthase (FAS) and polyketide synthase (PKS) of *Streptomyces glaucescens*. Initiation of straight-chain fatty acid biosynthesis by the type II FAS involves a direct condensation of acetyl-CoA with this malonyl-ACP to generate a 3-ketobutyryl-ACP product and is catalyzed by FabH. In vitro experiments with a reconstituted type II PKS system in the absence of FabH have previously shown that the acetyl-ACP (generated by decarboxylation of malonyl-ACP), not acetyl-CoA, is used to initiate tetracenomycin C (TCM C) biosynthesis. We have shown that sgFabH activity is present in *S. glaucescens* fermentations during TCM C production, suggesting that it could contribute to initiation of TCM C biosynthesis in vivo. Isotope incorporation studies with [CD_3]acetate and [$^{13}CD_3$]-acetate demonstrated significant intact retention of three deuteriums into the starter unit of palmitate and complete washout of deuterium label into the starter unit of TCM C. These observations provide evidence that acetyl-CoA is not used directly as a starter unit for TCM C biosynthesis in vivo and argue against an involvement of FabH in this process. Consistent with this conclusion, assays of the purified recombinant sgFabH with acetyl-CoA demonstrated activity using malonyl-ACP generated from either FabC (the *S. glaucescens* FAS ACP) (k_{cat} 42.2 min^{-1}, K_m 4.5 ± 0.3 μM) or AcpP (the *E. coli* FAS ACP) (k_{cat} 7.5 min^{-1}, K_m 6.3 ± 0.3 μM) but not TcmM (the *S. glaucescens* PKS ACP). In contrast, the sgFabD which catalyzes conversion of malonyl-CoA to malonyl-ACP for fatty acid biosynthesis was shown to be active with TcmM (k_{cat} 150 min^{-1}, K_m 12.2 ± 1.2 μM), AcpP (k_{cat} 141 min^{-1}, K_m 13.2 ± 1.6 μM), and FabC (k_{cat} 560 min^{-1}, K_m 12.7 ± 2.6 μM). This enzyme was shown to be present during TCM C production and could play a role in generating malonyl-ACP for both processes. Previous demonstrations that the purified PKS ACPs catalyze self-malonylation and that a FabD activity is not required for polyketide biosynthesis are shown to be an artifact of the expression and purification protocols. The relaxed ACP specificity of FabD and the lack of a clear alternative are consistent with a role of FabD in providing malonyl-ACP precursors for PKS as well as FAS processes. In contrast, the ACP specificity of FabH, isotope labeling studies, and a demonstrated alternative mechanism for initiation of the PKS process provide unequivocal evidence that FabH is involved only in the FAS process.

Streptomyces spp. utilize a type II, or dissociable, fatty acid synthase to catalyze formation of fatty acids (*1*, *2*). In this process a ketoacyl acyl carrier protein (ACP)[1] synthase (KASI, FabB) catalyzes a decarboxylative condensation of an acyl-ACP substrate and malonyl-ACP (Figure 1). Processing of the resulting 3-ketoacyl-ACP product by α-keto reduction, dehydration, and enoyl reduction steps leads to an extended acyl-ACP product, which serves as a substrate for a subsequent elongation by FabB. Initiation of fatty acid biosynthesis is catalyzed by a separate KAS (KASIII, FabH) catalyzing a direct condensation of an acyl-CoA thioester substrate with malonyl-ACP. The use of acetyl-CoA gives rise to straight-chain fatty acids while isobutyryl-CoA and methylbutyryl-CoA generate branched chain fatty acids (*3*, *4*). The malonyl-ACP extender unit used by FabB and FabH is generated from malonyl-CoA by the action of malonyl-CoA:ACP transacylase (MCAT, FabD). In *Streptomyces coelicolor* and *Streptomyces glaucescens* the four genes, *fabD*, *fabH*, *fabC* (encoding the ACP), and *fabB*, are clustered and appear at least in the case of *S. coelicolor* to be cotranscribed (*1*, *2*, *5*).

S. glaucescens and *S. coelicolor*, like many streptomyces, also contain type II or aromatic polyketide synthases. The type II PKS contains ketoacyl-ACP synthases (KSα and KSβ) which generate a 3-ketoacyl-ACP product in an manner analogous to that of FabB but using malonyl-ACP generated from a PKS ACP rather than a FAS ACP. Unlike the type II FAS the 3-keto group is not reduced after each condensa-

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[1] Abbreviations: ACP, acyl carrier protein; ACT, acetyl-CoA:ACP transacylase activity; FadA, thiolase; FabB, 3-ketoacyl-ACP synthase I (KASII); FabC, the *S. glaucescens* FAS ACP; FabD, malonyl-CoA:ACP transacylase (MCAT); FabH, 3-ketoacyl-ACP synthase III (KASIII); FAS, fatty acid synthase; IPTG, isopropyl β-D-1-thiogalactopyranoside; PKS, polyketide synthase; PMSF, phenylmethanesulfonyl fluoride; S-Gal, 3,4-cyclohexenoesculetin β-D-galactopyranoside; sgFabH, the *S. glaucescens* FabH; TCM C, tetracenomycin C; TcmM, the *S. glaucescens* PKS ACP; X-Gal, 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside.

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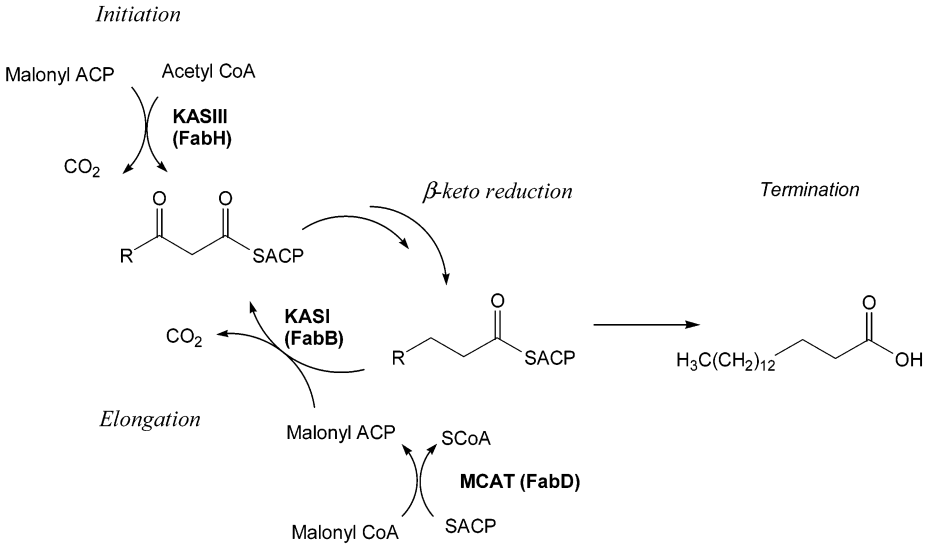

FIGURE 1: Role of FabH (KASIII), FabD (MCAT), FabC (ACP), and FabB (KASI) in generating a straight-chain fatty acid from acetyl-CoA and malonyl-CoA in a typical type II fatty acid synthase. FabC is the likely ACP used in all of these processes. The R group is a methyl substituent from the first FabH-catalyzed reactions. In the subsequent KASI-catalyzed reactions the R group is extended by two carbons.

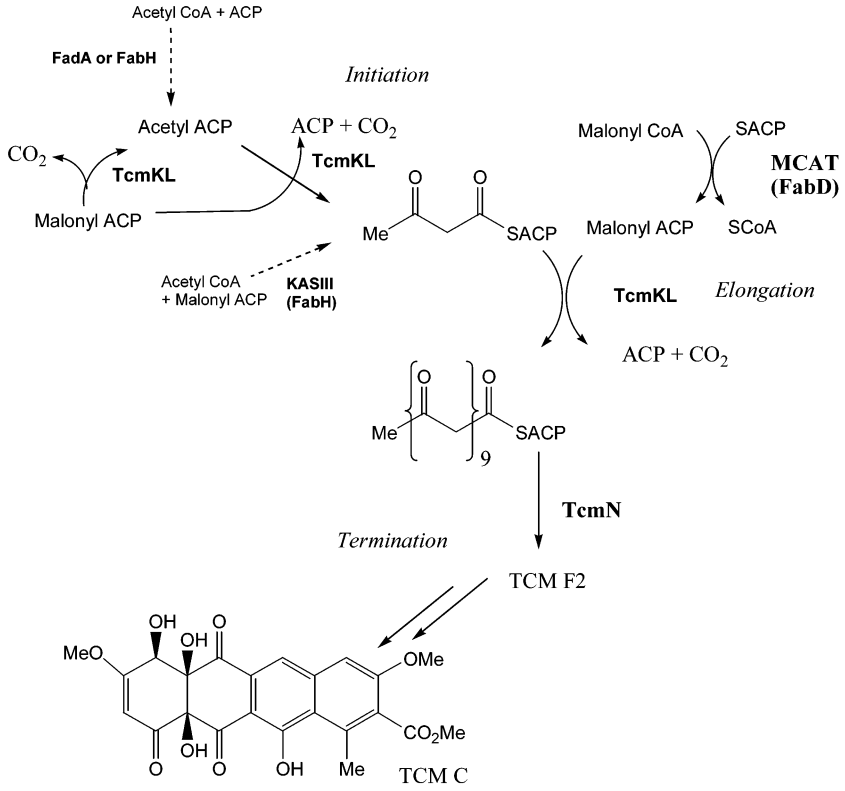

FIGURE 2: Role of TcmKL and TcmN in generating TCM F2 from acetyl-CoA and malonyl-CoA. Putative catalytic roles for FadA and the type II FAS component FabH are shown with dashed arrows and in smaller font. Eight cycles of the TcmKL-catalyzed extension of the 3-ketobutyryl-ACP substrate provide the polyketide substrate for TcmN. TcmM, the likely ACP used in all of these processes, has previously been proposed to be able to self-malonylate in the absence of FabD.

tion process, generating a polyketide product as opposed to a highly reduced fatty acid (Figure 2). In this way the minimal *S. glaucescens* tetracenomycin (TCM) PKS comprised of the KSα, KSβ (TcmK and TcmL), and the dedicated PKS ACP (TcmM) utilize malonyl-ACP and an acetate-derived starter unit to generate a decaketide product (Figure 2) (*6*). This product is first converted to TCM F2 by the action of TcmN and eventually to TCM C by additional enzymes. In vitro experiments with a reconstituted TCM PKS have shown that TcmKL can also decarboxylate malonyl-ACP to acetyl-ACP and use this as a starter unit but cannot utilize acetyl-CoA directly (*6*). Similar observations have been made for purified components from the *S. coelicolor* actinorhodin (ACT) minimal PKS (*7*). As is typical of bacterial antibiotic pathways the *S. glaucescens* TCM biosynthetic genes, including *tcmK*, *tcmL*, and *tcmM*, are clustered (*8*, *9*).

The similarities between the type II FAS and PKS and the processes they catalyze have raised the possibility that certain FAS components may play a role in both processes

(*7*). In particular, it has been argued that FabD provides the malonyl-ACP extender units used for both PKS and FAS (*1*, *2*, *6*, *7*). Most type II PKS clusters including the TCM PKS do not contain a *fabD* homologue, suggesting that the FAS FabD may catalyze this step (Figure 2). It has been demonstrated clearly that the *S. glaucescens* FabD can utilize both TcmM and FabC with comparable efficiency (*6*, *10*). It has also been shown that FabD is required for efficient production of the TCM F2 decaketide precursor from malonyl-CoA using TcmKL, TcmM, and TcmN (*6*). Finally, an absolute FabD requirement has been observed for production of an octaketide product from the purified components of the *S. coelicolor* minimal ACT PKS (*7*). In the latter case it has also been reported that, under conditions where the PKS ACP is present in excess as compared to the KS components, FabD is not an absolute requirement (*11*). This latter observation is consistent with the observation that the ACT *holo*ACP, TcmM, and other PKS ACPs can self-malonylate using malonyl-CoA (*10*, *12*). The role of FabD in aromatic polyketide biosynthesis, and the basis for these contradictory and controversial observations, has remained elusive.

A role for FabH in initiation of polyketide biosynthesis has also been suggested (*1*) but never probed. FabH-catalyzed condensation of acetyl-CoA with malonyl-ACP generated from the PKS ACP would generate the first 3-ketobutyryl-ACP intermediate in the polyketide biosynthetic pathway (Figure 2). Such a process would allow acetyl-CoA to be used directly and circumvent the in vivo carboxylation−decarboxylation initiation process inferred from studies of TCM C and ACT biosynthesis with a reconstituted minimal PKS (FabH was not included in these studies) (*6*, *7*, *11*).

We report herein that while FabH is present in *S. glaucescens* during TCM C production, feeding studies with labeled acetate clearly demonstrate that it does not play a significant role initiating the biosynthesis process from acetyl-CoA. Consistent with this in vivo observation, the purified sgFabH is shown to have a remarkable ACP specificity for FabC over TcmM. In contrast, the sgFabD is also active during TCM C production in *S. glaucescens* fermentations but does not differentiate significantly between TcmM and FabC. Previous descriptions of the ability of TcmM and other PKS ACPs to self-malonylate are shown to be an artifact of the purification process, leading to conclusion that, by default, FabD plays a role in both FAS and PKS processes. These observations also demonstrate that ACP specificity of the FAS FabH and likely the PKS KSα, KSβ serves as the branch point that separates these two processes and the likely reason for previous in vivo observations which demonstrated that the FAS ACP cannot be used for polyketide biosynthesis in the absence of the PKS ACP (*13*).

MATERIALS AND METHODS

Expression and Purification of TcmM Using Escherichia coli and Baculovirus Expression Systems. The expression plasmids containing genes for *S. glaucescens* N-terminal polyhistidinyl TcmM and holo-acyl carrier protein synthase (ACPS) were kindly provided by C. T. Walsh, Harvard Medical School. Purified FrnN was provided by C. Khosla, Stanford. Protein expression in *E. coli* cells, modification of apo-TcmM by ACPS, and purification of holo-TcmM were carried out as described previously (*10*). Additional purification of holo-TcmM was carried out using a Mono Q HR5/5 column using a 0−500 mM NaCl at flow rate 1 mL/min in buffer containing 100 mM $NaHPO_4$, 2 mM EDTA, and 2 mM DTT at pH 7.3. Fractions containing the TcmM were identified by native PAGE.

The pFastBac Dual expression vector (Life Technologies, Inc.) was used to obtain simultaneously expressed TcmM and ACPS in insect cells. To amplify *acpS*, the following PCR primers were used: 5′-GAAGGAGATATACCATGG-CAATATTAGG-3′ [rightward primer mapping on the upstream sequence of *acpS* and having an *Nco*I site (underlined) introduced to facilitate cloning] and 5′-GTGCGGCCGC-GCATGCTTAACTTC-3′ [leftward primer mapping on the downstream sequence from *acpS* and having an *Sph*I site (underlined) introduced to facilitate cloning]. The *Nco*I−*Sph*I-digested PCR product was cloned into expression vector pFastBac Dual to give pGF201. The *tcmM* gene was PCR amplified using the following primers: 5′-AGAAGGAGA-TCTAGATATGCCCCAGA-3′ [rightward primer mapping on the upstream sequence of *tcmM* and having an *Xba*I site (underlined) introduced to facilitate cloning] and 5′-CAGCTT-CCTTTCAAGCTTTGTTAGC-3′ [leftward primer mapping on the downstream sequence from *tcmM* and having a *Hin*dIII site (underlined) introduced to facilitate cloning]. The *Xba*I−*Hin*dIII-digested PCR product was cloned into the corresponding sites of pGF201 to give pGF202. The pGF202 was used for transposition into the mini-attTn7 target site of the bacmid by electroporation of DH10Bac cells and plated on LB−ampicillin plates containing X-Gal or S-Gal (100 μg/mL) and IPTG (40 μg/mL). White colonies were verified by streaking onto fresh plates, and five were selected for analysis. The presence of each insert in bacmid DNA was verified by PCR using gene-specific primers.

The resulting recombinant bacmid DNA was introduced into SF9 cells by lipofection (CELLFECTIN reagent, Life Technologies, Inc.). Primary virus stock was harvested 72 h posttransfection, and infected cells were analyzed for protein production. Virus was amplified by infecting log phase 100 mL cultures with primary virus stock and harvesting 30−40 h later. This process was repeated until high titer virus stocks were achieved (1×10^8 pfu/mL). The time course experiments revealed optimal expression in cells harvested 48 h after infection at a multiplicity of infection of 1. Viral stocks were harvested and used to infect fresh insect cells for subsequent protein expression, and purification yielded approximately 10 mg/L recombinant TcmM (the yield of ACPS has not been determined).

The His-tagged TcmM obtained from the insect cells, like that obtained from *E. coli*, was purified by affinity and gel-filtration chromatography as described previously (*10*). The TcmM was then assayed for malonylation in the presence and absence of FabD without additional purification steps. The purity of the TcmM was estimated by native PAGE.

Modification of Apo-TcmM to Holo-TcmM. Using conditions described previously the apo form of TcmM purified from *E. coli* was modified to holo-TcmM (using coenzyme A) or acetyl-TcmM (using acetyl-CoA) by taking advantage of the broad substrate specificity of ACPS (*10*). An HPLC assay was used to confirm the ACP modification (>95%) using as a solvent system a 15−75% acetonitrile gradient in 0.1% trifluoroacetic acid.

Analyses by HPLC revealed that the TcmM product obtained from insect cells contained primarily holo-ACP (approximately 70%) and a minor amount of apo-ACP (30%). Incubation of this affinity-purified TcmM with CoA, as previously described (*10*), led to a complete modification of TcmM as judged by HPLC. The subsequent gel-filtration step provided the purified holo-TcmM.

Expression and Purification of FabD. The *S. glaucescens* FabD was expressed and purified from *E. coli* as described previously (*10*).

Effect of ACP on FabD Inhibition by PMSF. Purified AcpP (*E. coli*, Sigma Chemical Co.) was reacted with PMSF prior to incubation with malonyl-CoA as described previously-(*10*). FabD (0.1 μM) was incubated with PMSF (2 mM) at 30 °C for 30 min at pH 7.5, prior to conducting a standard assay. Control experiments were carried out without addition of PMSF. An additional series of studies adding ACP (12 and 24 μM) prior to PMSF was also carried out.

Enzyme Assays. The FabD and FabH assays were carried out as described previously (*3*, *10*). Malonyl-ACP for FabH assays was prepared as described previously (*14*). The ACT (acyl-CoA:ACP transacylase) assay was carried out as described previously (*3*). Resolution of FabH and FadA activities in cell-free extracts was accomplished using a Sephadex G-100 column (2.5 × 100 cm) (*15*).

Production and Quantification of TCM C. A two-stage fermentation process was used for TCM C production. At the first stage an *S. glaucescens* spore suspension was used to inoculate liquid fermentation medium (*16*) and subsequently incubated at 30° C for 24 h. A 10% inoculum of this seed culture was transferred into the second stage fermentation containing the same medium and incubated under the same conditions for an additional 96 h. Samples of the culture (1 mL each) were withdrawn at 15, 24, 48, 52, and 72 h of fermentation, cells on ice were repeatedly sonicated for a short period of time (10−15 s), and cellular debris was removed by centrifugation to generate cell extracts for FabD, FabH, and ACT assays. TCM C was extracted from the acidified samples (glacial acid, 20 μL/mL of culture) with the same volume of ethyl acetate and subjected to TLC and HPLC assays (*16*, *17*). Total TCM C titers were quantified by comparison of the integrated peak areas against the standard curve obtained with a previously purified sample of TCM C.

Production of TCM C and Isotope Incorporation Studies. An *S. glaucescens* fermentation (5 L) grown for 96 h in fermentation medium (*16*) was acidified and extracted with ethyl acetate (2 × 5 L). The extract was concentrated, and the pellet was washed with petroleum ether and further purified by flash chromatography as described previously (*17*). Pooled fractions of TCM C were further purified by semipreparative HPLC on a MetaSil C18 column (5 μm, 250 mm × 10 mm). The column was developed with a 100 mL linear gradient of acetonitrile/H_2O from 20:80:0.1 to 80:20:0.1 in 0.1% glacial acid at a flow rate of 5 mL/min. TCM C eluted with an approximate retention time of 13 min. Pooled fractions were dried under vacuum and were subjected to analysis by ^{1}H and ^{13}C NMR.

In the isotope incorporation studies, conditions for production and isolation of TCM C were identical to those described above. A 1:3 mixture of [2-^{13}C,$^2H_3$]acetate and unlabeled acetate was prepared at pH 7.4 and added batchwise at 24, 36, and 48 h during the production phase of *S. glaucescens* fermentation to a final concentration of 30 mM. The purified TCM C sample (10 mg) was subjected to ^{2}H,^{1}H-decoupled and ^{1}H-decoupled ^{13}C NMR spectroscopy as previously described (*18*).

Fatty Acid Analysis and Incorporation Experiments with [2-$^2H_3$]Acetate. *S. glaucescens* cultures were grown in minimal or fermentation media at 30 °C for 49 h, and the fatty acids were extracted and analyzed as described previously (*19*, *20*). A 1:3 mixture of [2-$^2H_3$]acetate and unlabeled acetate (30 mM) was prepared at pH 7.4 and added as a single dose to the fermentation media at either 0 or 30 h of incubation.

Kinetic Determinations with FabD. The apparent K_m and k_{cat} values of FabD for TcmM purified from insect cells were determined under standard 30 s assay conditions using 50 μM [2-^{14}C]malonyl-CoA (50000 cpm) and variable concentrations of the ACP substrates. To account for any loss in FabD activity and variations in specific activity, control experiments with the *E. coli* AcpP were conducted simultaneously and used to normalize the k_{cat} for malonylation of different ACPs. The simultaneous assays were also carried out in the absence of FabD, allowing the process of self-malonylation to be followed. All reactions and controls were performed at least in triplicate. All kinetic values for FabH and FabD were obtained by nonlinear regression analysis using GraFit 4.0 (Erithacus Software).

Determination of the N-Terminal Amino Acid Sequence of the TcmM Purified from Insect Cells. The purified holo-TCM C obtained from insect cells was electrophoresed on a 20% native PAGE gel and then blotted onto a PVDF membrane (Sequi-Blot, Bio-Rad) using a Hoefer SemiPhor transfer blotter (Amersham). The membrane was stained with Coomassie Stain solution (Bio-Rad), and the region containing the protein band was used for N-terminal amino acid analysis by Edman degradation (Pfizer, Inc.).

RESULTS AND DISCUSSION

FadA, FabH, and FabD Activities Are Present in S. glaucescens during TCM C Production. If either FabH or FabD contribute to TCM C biosynthesis, they must be present during and after late log growth. The *S. glaucescens* FabC is present 6 h earlier in growth than TcmM but is still the major ACP at late log phase of growth (*2*). The presence of FabC during TCM C production led to the argument that FabD and other components of the type II FAS may be also present, as *fabDHCB* genes are likely cotranscribed (*2*). Subsequent analysis of *S. coelicolor* supports cotranscription of these genes at least until mid log phase growth (*5*). Expression of streptomycete type II FAS components during late phase growth and their relative stability have not been previously determined. Thus the activity of FabH and FabD during TCM C production remained an open question. A similar situation is also presented with the thiolase (FadA) enzyme. This enzyme purified from *Streptomyces collinus* has been shown recently to have a unique acetyl-CoA:ACP transacylase activity (ACT) (*15*), using both PKS and FAS ACPs. The presence of an enzyme activity during TCM C production would support the possibility of an additional pathway from initiation of biosynthesis involving FadA-catalyzed conversion of acetyl-CoA to acetyl-ACP using TcmM (Figure 2).

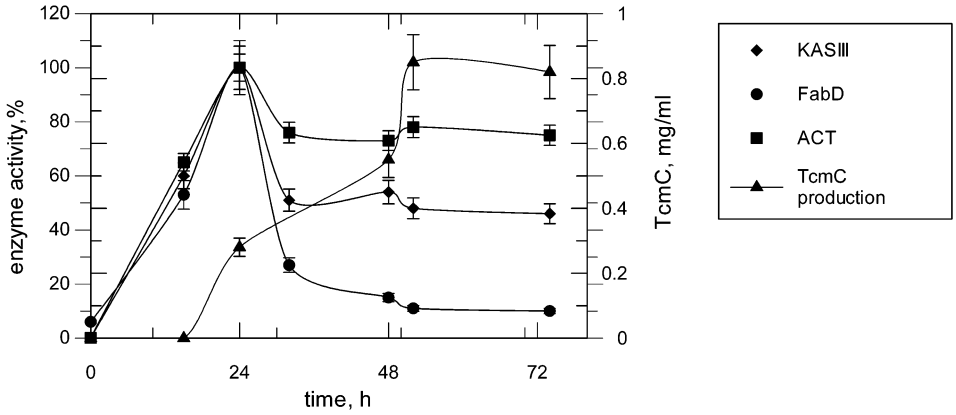

FIGURE 3: Enzyme activity and TCM C production levels over the time course of fermentation of *S. glaucescens.* Each enzyme activity is described as a percentage of the maximal level obtained from examination of cell-free extracts. Data represent duplicate enzyme assays carried out on the same cell-free extract assays generated from a single fermentation study. Analyses from additional fermentation studies did not differ qualitatively from these.

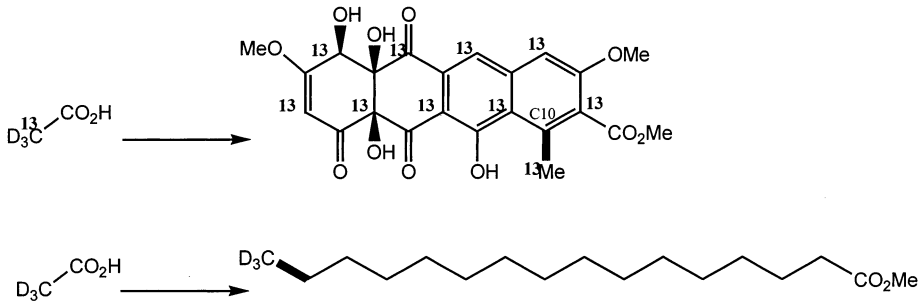

FIGURE 4: Schematic representing incorporation of $^{13}CD_3$- and $^{12}CD_3$-labeled acetate into TCM C and palmitate, respectively. A bolded bond indicates the acetate-derived starter unit.

We examined the levels of FabD, FabH, and ACT activities during *S. glaucescens* fermentation. As shown in Figure 3 TCM C production was detected by 24 h of fermentation and reached a maximum at around 52 h. FabD activity reached maximal activity at around 24 h of growth and then decreased to 10% of this level by 72 h. FabH and ACT activity also reached maximum levels at 24 h and decreased to approximately 45% and 70% of this level by 72 h, respectively. As ACT activity is a property of FadA and FabH (*3*), these assays with cell-free extracts did not allow us to unequivocally demonstrate if FadA is present during the later states of the fermentation process. We have previously reported that the homotetrameric FadA of *S. collinus* is readily separable from the smaller homodimeric FabH by gel exclusion chromatography (*15*). In the same way, gel exclusion chromatography of an *S. glaucescens* fermentation at 72 h revealed the presence of clearly resolved peaks for ACT activity of these two proteins (data not shown). Numerous factors, including protein stability and the inhibitory effects of other components in the cell extracts, may contribute to the observation that levels of FabD activity decrease more rapidly after 24 h than of FabH and ACT activity. Nonetheless, this preliminary analysis provides the first clear evidence that all three enzymatic activities are still present in *S. glaucescens* during TCM C production.

Incorporation Studies with [2-^{13}C,$^{2}H_3$]- and [2-$^{2}H_3$]Acetate Demonstrate a Direct Pathway for Initiation of Straight-Chain Fatty Acid Biosynthesis but not TCM C from Acetyl-CoA. We have previously shown that addition of perdeuterated acetate to fermentations of *S. glaucescens* and *S. collinus* results in the generation of the palmitate containing three deuteriums in the ω-methyl group (*20*, *21*) (Figure 4). Such compounds (with a molecular ion of 273) are readily detected by a GC−MS analysis where they exhibit a slightly shorter retention time than the corresponding unlabeled methyl palmitate (with a molecular ion of 270) (*19*, *20*). These results are consistent with the perdeuterated acetate being converted in vivo to perdeuterated acetyl-CoA and competing with endogenous acetyl-CoA for initiation of palmitate biosynthesis by FabH (*21*). There is no significant washout of label in this process and between 10% and 20% of the palmitate pool can be readily labeled with three deuteriums. In contrast, there is poor labeling of palmitate by a malonyl extender unit for several reasons, including loss of deuterium in the conversion of perdeuterated acetyl-CoA to malonyl-CoA and deuterium washout at the level of malonyl-CoA/malonyl-ACP (*4*). An indirect pathway for initiation of TCM C biosynthesis involving a carboxylation−decarboxylation process and malonyl thioester intermediate (Figure 2) would, therefore, be predicted to lead to significant loss of deuterium at the starter unit, in clear contrast to results observed with initiation of palmitate biosynthesis.

In this study we used [2-^{13}C,$^{2}H_3$]acetate to follow the fate of hydrogens attached to acetyl-CoA as they were incorporated into TCM C. Approximately 10 mg of pure TCM C was obtained from a 5 L fermentation of *S. glaucescens* in the presence of 7.5 mM [2-^{13}C,$^{2}H_3$]acetate and 22.5 mM unlabeled acetate. A ^{13}C NMR experiment with deuterium and proton decoupling demonstrated comparable (4.1−5.5-fold) enrichment of the appropriate carbons of TCM C (Figure 4). No evidence for a ^{2}H-shifted peak was observed for any of the TCM C assigned peaks, including the C10

methyl resonance at 21.0 ppm. No changes were observed to any of the TCM C resonances when the sample was run in the absence of deuterium decoupling. These observations support the use of acetate for both the starter and extender units for TCM C biosynthesis with extensive washout of deuterium label, consistent with the presence of a malonyl thioester intermediate in both processes. Such an observation is inconsistent with the use of either FabH or FadA to initiate TCM C biosynthesis directly from acetyl-CoA (Figure 2). A similar lack of intact incorporation of deuterium label from [2-^{13}C,^{2}H$_3$]acetate has been reported for the starter unit of granaticin (*22*). This observation led to a suggestion of malonyl-CoA as a starter unit, which is decarboxylated by the "enzyme matrix". In the granatacin study NMR analysis of the methyl group derived from the starter unit demonstrated no evidence of ^{13}C^{2}H$_3$ but some level of ^{13}C^{2}H$_2$ and ^{13}C^{2}H species (*22*). In our study on TCM C we did not see any evidence for these species. In contrast to these results intact incorporation of [2-^{13}C,^{2}H$_3$]acetate has been previously observed in the fungal polyketide product cladosporin and oleic acid in *Cladosprium cladosporoides*, suggesting that in this case neither process initiates with decarboxylation of a malonyl thioester starter unit (*23*).

We confirmed intact incorporation of [2-^{2}H$_3$]acetate into palmitate in the *S. glaucescens* fermentation media under conditions comparable to that used for the TCM C labeling study. [2-^{13}C,^{2}H$_3$]Acetate was not used in this study because incorporation of the ^{13}C label into the malonate-derived positions masked intact labeling of the starter unit with deuterium. When a mixture of labeled acetate and unlabeled acetate was added at 0 h to the fermentation, we observed approximately 12% intact labeling of the starter unit with three deuteriums, consistent with previous observations (Figure 4) (*4*). Approximately 3% of each of the malonate-derived positions were monodeuterated. Much lower levels of labeling (less than 1%) of both the starter unit and extender units were observed when feeding was carried out at 30 h, consistent with the majority of fatty acids having been generated. As the differential timing of fatty acid and TCM C production requires that these feeding experiments cannot be carried out under identical conditions, we cannot exclude the possibility that this may account for intact retention of deuterium from perdeuterated acetate into the starter unit of palmitate but not TCM C. However, the data from these and previous studies are all consistent with recent in vitro analyses that suggest TCM C and other similar type II PKSs initiate biosynthesis by catalyzing decarboxylation of malonyl-ACP.

The S. glaucescens FabH Effectively Discriminates between a FAS (FabC) and PKS (TcmM) ACP. It has previously been observed that replacement of the actinorhodin ACP gene (*actI* ORF3) by the corresponding FAS ACP gene (*fabC*) in *S. coelicolor* leads to a significant drop in actinorhodin production (*13*). A PKS ACP specificity may contribute to this observation, and this possibility could now be tested in vitro with the availability of reconstituted purified PKS systems for kinetic analyses of polyketide production. Similarly, a specificity of the sgFabH for a FAS ACP may explain why the enzyme is present in *S. glaucescens* during TCM C production but has no apparent role in initiating the process. We tested this hypothesis by evaluating the activity of the sgFabH using the three different ACPs (Table 1) and

Table 1: Kinetic Values for Reactivity of AcpP, FabC, and TcmM with the *S. glaucescens* FabH and FabD[a]

enzyme	ACP substrate (FabD) and malonyl-ACP substrate (FabH)	k_{cat} (min^{-1})	K_m (μM)	k_{cat}/K_m (μM^{-1} min^{-1})
FabD	AcpP (*E. coli*)	141	13.2 ± 1.6	10.7
	FabC (*S. glaucescens*)	560	12.7 ± 2.6	44.0
	TcmM (*S. glaucescens*)	150	12.2 ± 1.2	12.3
FabH	AcpP (*E. coli*)	7.5	6 ± 0.3	1.26
	FabC (*S. glaucescens*)	42.2	4.5 ± 0.3	9.4
	TcmM (*S. glaucescens*)	<0.0001	NA[b]	NA[b]

[a] Kinetic values were determined using 30 s assays. A calculation error in a previous preliminary analysis of k_{cat} values of FabD (*10*) resulted in these values being underreported. K_m values reported here are calculated using the Lowrie method for quantification of the ACP substrates. In a previous report (*10*) the use of a Bradford assay underestimated ACP quantities leading to higher reported apparent K_m values. [b] Not active.

acetyl-CoA. The sgFabH was able to react with both FAS ACP from *E. coli* (AcpP) and FabC (FabC) but consistently had no detectable activity with TcmM, the PKS ACP. We observed that the TcmM sample used in these FabH assays was active both with malonyl transacylase activity of the *S. glaucescens* FabD (Table 1) and with the acetyl transacylase activity of the *S. collinus* FadA (*15*). The sgFabH thus effectively discriminates between the FAS ACP and PKS ACP from the same organism. Malonyl-ACP generated in *S. glaucescens* using TcmM is thus precluded from being used by the sgFabH. This observation predicts that, for a FabH activity to function in a polyketide biosynthetic process, it would need to have a different ACP specificity. Certain aromatic polyketides do not utilize malonyl-CoA as a starter unit but propionyl-CoA (danorubicin), isobutyryl-CoA (R1128), or butyryl-CoA (frenolicin) using FabH homologues. Analysis of the corresponding PKS gene clusters has revealed that in addition to FabH homologue there are sometimes two PKS ACPs (*24*, *25*). Presumably the FabH homologue in these systems is able to react with at least one of these PKS ACPs. Indeed, recent evidence from in vitro inhibition experiments with apo forms of the two PKS ACPs (ZhuG and ZhuN) encoded by the R1128 PKS gene cluster suggests that the corresponding holo forms are comparable substrates for the FabH homologue ZhuH, which initiates this biosynthetic process. It is also possible that ZhuH and other analogous FabH activities associated with aromatic PKS biosynthesis may discriminate against a FAS ACP.

A comparison of the k_{cat}/K_M values for the sgFabH revealed that it is 7-fold more active with the malonyl-ACP generated from FabC (*S. glaucescens*) than AcpP (*E. coli*). Previous enzymatic analyses of FabH enzymes from other bacteria including *Mycobacterium tuberculosis* (*26*, *27*), *Bacillus subtilis* (*28*), and *Streptococcus pneumoniae* (*29*) have been carried out using commercially available *E. coli* ACP. In many cases the activity of these proteins is significantly lower than that reported for the *E coli* FabH (*28*, *29*), and it is now reasonable to suggest that they may also be more efficient when presented with the physiologically relevant ACP.

A model for interaction of the *E. coli* FabH with the cognate AcpP has been proposed on the basis of computational analysis and supported by mutational studies (*30*). In

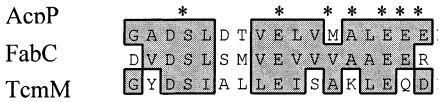

FIGURE 5: Alignment of the α-helix of AcpP, FabC, and TcmM. The asterisk indicates AcpP residues proposed to be important in forming an interaction with the ecFabH.

this model, the closest contact between the proteins is of helix II of AcpP and helix Cα2 of FabH, which are oriented at an angle of −60 °C. Differences in helix II of different TcmM and FabC, therefore, might account for the ability of the sgFabH to react only with FabC. It has been previously reported that the region from Leu32 to Phe52, which includes this helix and the prosthetic group attachment site (Ser 36), is highly conserved (*30*). A comparison of the α-helix region of AcpP with FabC and TcmM (Figure 5), however, revealed several significant differences (Figure 5). Three of the four glutamate residues (Glu 41, 47, 48, and 49) in AcpP, which are predicted to form favorable interactions with the ecFabH, are conserved in the FabC. In the case of TcmM only two of these glutamates are present. Similarly, the AcpP Met 44 is proposed to contribute toward interaction with the ecFabH and, conserved among many ACPs, is not present in either TcmM or FabC. Mutational analyses will provide an understanding of whether any of these differences contribute to the preference of sgFabH for FabC.

In contrast to the sgFabH we have previously shown that the ACT activity of *S. collinus* FadA does not discriminate between ACPs (*15*). The FadA type activity shown to be present in *S. glaucescens* during TCM C production (see above) may have similar properties. Nonetheless, the labeling studies preclude FadA from a significant role for in vivo initiation of TCM C biosynthesis. The ACT activity of FadA is only about 1/1000 of its thiolase activity and is strongly inhibited by coenzyme A (*15*). We have shown that significant pools of coenzyme A are present in a variety streptomycete cell extracts (Florova and Reynolds, unpublished data), suggesting that under a number of growth conditions the ACT activity of FadA may not have a significant physiological role.

TcmM Expressed and Purified from Insect Cells Does Not Self-Malonylate. In the majority of experiments on in vitro reconstitution of minimal PKS and analysis of substrate specificity of FabD (*7*, *11*, *12*, *31*), the apo-ACP or holo-ACP was expressed and purified as a recombinant protein from *E. coli* cells. The TcmM has been expressed in both *Streptomyces lividans* (*6*) and *E. coli*. While the use of these expression systems allows a high level of the required protein to be readily obtained, they carry a disadvantage that the host system carries a highly active FabD. A minor contamination by FabD could account for the observation that many ACP preparations appear to self-malonylate (*11*, *12*, *31*).

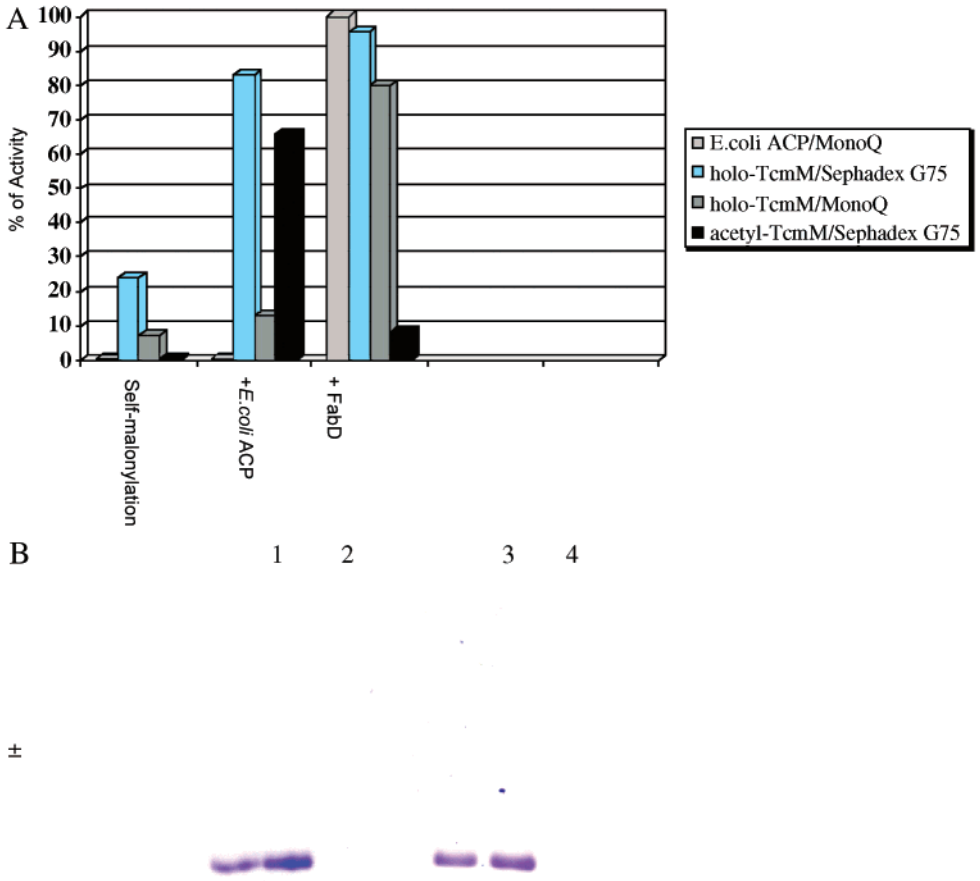

FIGURE 6: (A) Effect of an additional purification step on the ability of holo-TcmM and acetyl-TcmM samples to catalyze apparent self-malonylation and malonylation of *E. coli* ACP. The same concentrations (4 μM) of AcpP and holo-TcmM were used in these 7 min assays. Assays were conducted either with just malonyl-CoA (self-malonylation) or with addition of malonyl-CoA and either *E. coli* AcpP (6 μM) or FabD (0.1 μM). Relative malonylation rates are expressed relative to that observed with the FabD-catalyzed reaction with TcmM and AcpP. (B) Native PAGE demonstrating apparent purity of pooled fractions during TcmM purification. Lanes: holo-TcmM purified sequentially using Ni-NTA (lane 1), Sephadex G-75 (lane 2), and Mono Q (lane 3) chromatography; acetyl-TcmM purified using Ni-NTA and Sephadex G-75 chromatography (lane 4).

Evidence against a FabD contamination has been the apparent purity of the PKS ACP samples and the observation that PMSF (which inhibits FabD) has no effect on the rate of malonyl transfer by purified PKS ACPs (*12*). In an attempt to clarify this issue, we took a TcmM sample that was apparently pure after affinity chromatography and size exclusion chromatography and subjected it to additional purification steps. The ability of holo-TcmM to catalyze apparent self-malonylation and malonylation of AcpP was then analyzed (Figure 6). The TcmM after the Sephadex G-75 or Mono Q purification steps reacted with sgFabD (0.1 μM) exhibited levels of malonylation comparable to that observed with purified AcpP in a 7 min assay. As previously reported, the holo-TcmM (4 μM) sample purified by Sephadex G-75 and affinity chromatography also catalyzed an efficient apparent malonylation of the AcpP in the absence of FabD under the same assay conditions (the AcpP was not malonylated in the absence of TcmM). At least a 4-fold decrease in malonylation of AcpP was observed when this assay was repeated with TcmM (4 μM) that had been purified by the additional Mono Q step. The additional Mono Q purification step led to a similar decrease in the ability of the TcmM to catalyze an apparent self-malonylation. These observations all suggested that the catalytic properties of TcmM may be attributable to a contaminating protein. Additional evidence was obtained by generating acetyl-TcmM (this was prepared from apo-TcmM using an advantage of a broad substrate specificity of ACPS). As predicted, this protein was neither a substrate for FabD nor able to catalyze self-malonylation (Figure 6). The acetyl-TcmM sample was, however, able to catalyze malonylation of AcpP at a rate comparable to that observed with the holo-TcmM. This observation is inconsistent with a mechanism involving transfer of the malonyl group from malonyl-CoA to AcpP via the sulfhydryl group on the TcmM prosthetic group (*10*) and again suggests that the catalytic activity is attributable to an impurity.

One argument against a FabD impurity has been the observation that the purity of ACP samples was determined as greater than 99% as judged by SDS−PAGE and ESMS (*12*). In the current study native PAGE analysis of the holo-TcmM shows an apparent identical level of purity after each purification step, and SDS−PAGE analysis does not reveal proteins within the expected mass range for FabD. Nonetheless, the additional purification step consistently leads to a decrease only in the apparent ability to either self-malonylate or malonylate AcpP but not be a substrate for FabD.

Concerns about FabD contamination have led to PKS ACP samples being preincubated in the presence of FabD inhibitor PMSF (*12*, *31*). This PMSF preincubation had no effect on the rate of malonylation by the active C17S holo-ACP, leading to the argument that such activity was not due to the presence of FabD (*12*). These experiments did not take into account any potential effects that large 25−50 μM concentrations of ACP may have on FabD inhibition. We investigated this possibility by initially confirming that an incubation of FabD (0.1 μM) with PMSF (2 mM, 30 min) at 30 °C led to greater than 98% loss of activity. When this preincubation with FabD was repeated in the presence of just 12 μM AcpP, more than 60% of the activity was retained. The most likely interpretation for this observation is that the low concentration of FabD is protected from PMSF inhibition by the high concentrations of substrate ACP. Similarly, low levels of a FabD contamination in PKS ACP samples would not be inhibited significantly by PMSF. Thus neither the apparent purity of PKS ACPs nor the use of preincubation with PMSF can conclusively rule out the possibility that FabD or some other enzyme accounts for the observed self-malonylation.

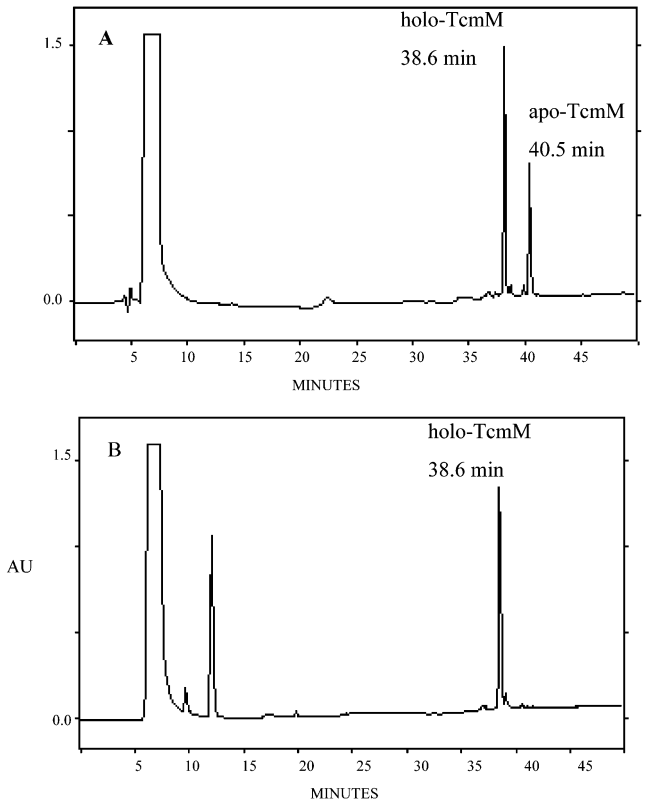

FIGURE 7: Conversion of a mixture of apo-TcmM (A) to holo-TcmM (B) by ACPS and coenzyme A. The TcmM and ACPS were simultaneously expressed using the pFastBac DUAL baculovirus expression system.

To resolve this controversial issue, we used an alternative baculovirus expression system pFastBac Dual. This system allowed simultaneous expression of ACPS and TcmM in insect cells, a host system that has no apparent type II FAS or FabD. The TcmM obtained from the insect cells was a mixture of the holo and apo forms, as determined by HPLC analysis (Figure 7). Incubation of the Ni-NTA-purified TcmM with CoA as previously described (*10*) led to a complete modification of TcmM as judged by HPLC (Figure 7). An N-terminal peptide sequence of the purified TcmM, coexpressed with ACPS using pFastBac Dual, revealed 100% identity to the TcmM. At any given concentration of TcmM (1−12 μM) in the absence of FabD, there was no significant malonylation in a standard 1 min assay (Figure 8). In contrast in the presence of the *S. glaucescens* FabD (0.1 μM) the same TcmM sample was readily malonylated (Figure 8), clearly demonstrating that the protein sample obtained from this different host expression system remains a substrate for this enzyme. As expected, higher levels of malonylated TcmM were observed as the concentration of TcmM was increased, but only in the presence of FabD. These observations contrast previous analyses using the same precipitation assays, which demonstrated that malonylation of TcmM and other PKS ACPs occurs in the absence of FabD (*2*) and is

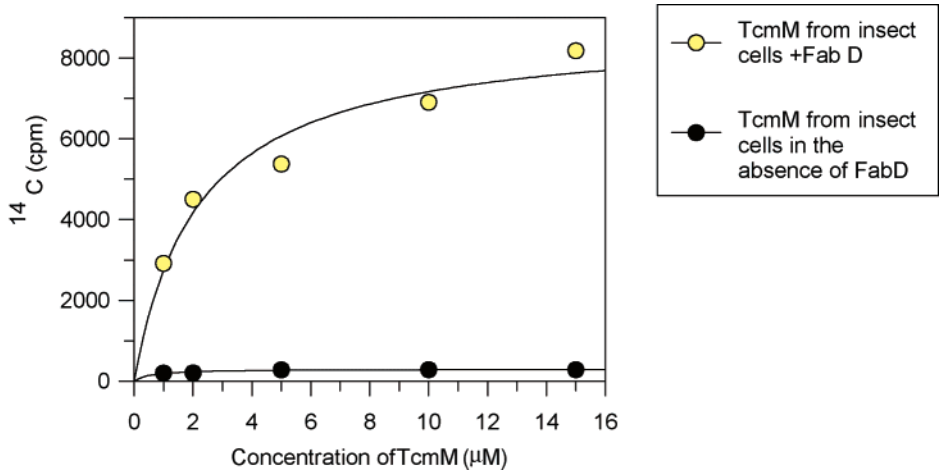

FIGURE 8: Effect on TcmM concentration on the rate of malonylation by malonyl-CoA occurs only in the presence of FabD (0.1 μM). The amount of ^{14}C-radiolabeled malonyl-ACP generated in the presence of ^{14}C-labeled malonyl-CoA is plotted (*Y*-axis) as a function of TcmM concentration (*X*-axis) and was determined in a 1 min assay as described in Materials and Methods.

dependent upon ACP concentration (*12*, *31*). In the case of the TcmM, a 30 min incubation of a 10 μM sample in the presence of 50 mM malonyl-CoA did not even lead to any significant level of malonylation. Equivocal observations to these made with TcmM obtained from insect cells were also made with FrnN (the PKS ACP used in frenolicin biosynthesis). This FrnN sample rigorously purified from *E. coli* has been shown to support actinorhodin biosynthesis in a reconstituted minimal PKS system only in the presence of FabD (*7*).

Taken together, this series of analyses provides compelling evidence that the apparent self-malonylation properties attributed to some PKS ACP samples are an impurity, most likely FabD.

ACP Substrate Specificity of FabD As Compared to FabH. Many type II aromatic polyketide biosynthetic gene clusters do not contain a FabD homologue, and PKS ACPs self-malonylation now appears to be an artifact. In cases such as actinorhodin and TCM C biosynthesis, the fatty acid biosynthetic FabD is the only identified catalyst which could provide the malonyl-ACP required for both initiation and subsequent elongation steps. Consistent with such a role, the FabD should be able to react with both the PKS and FAS ACPs (contrasting the properties observed for the FabH). Indeed, such a property has been shown for FabD from both *S. coelicolor* and *S. glaucescens* (*1*, *2*, *31*). An estimation of the kinetic properties of the *S. glaucescens* FabD indicated that AcpP, FabC, and TcmM were equivalent substrates (*31*). These kinetic determinations were hampered by TcmM self-malonylation and low quantities of the monomeric form of TcmM (*31*). The use of insect cells as an alternative expression system for generating TcmM overcomes these limitations and allows a more accurate kinetic comparison to be made. These analyses revealed that FabD as well as FabH had a clear preference for FabC over the *E. coli* AcpP (Table 1). The kinetic properties for the *S. glaucescens* FabD with FabC were comparable to those reported for the corresponding *E. coli* enzymes (*32*). The *S. glaucescens* FabD was also able to efficiently process TcmM with K_m values comparable to those observed with the FabC. These observations are consistent with the proposed role of FabD in generating malonyl-ACP for both TCM C and fatty acid biosynthesis. FabD processes FabC almost four times more efficiently than TcmM. In cases where *fabD* homologues are encoded within a bacterial aromatic biosynthetic gene cluster, the opposite may occur. Thus ZhuC involved in R1128 biosynthesis, which appears to react with the PKS ACPs (ZhuG and ZhuN), might do so more efficiently than with the corresponding FabC.

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