Polyketide synthase acyl carrier protein (ACP) as a substrate and a catalyst for malonyl ACP biosynthesis

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Background: Using an acyl–acyl carrier protein (ACP) as a starter unit, type II polyketide synthases (PKSs) generate a wide range of polyketide products by successive decarboxylative condensations with the two-carbon donor malonyl (ACP). *In vitro* experiments have demonstrated that polyketide biosynthesis in reconstituted PKS systems requires the fatty acid synthase (FAS) enzyme malonyl CoA:ACP acyltransferase (FabD) from streptomycetes. It has also been shown that *holo*-ACPs from a type II PKS can catalyze self-malonylation in the presence of malonyl CoA and negate this FabD requirement. The relative roles of FabD and ACP self-malonylation in PKS biosynthesis *in vivo* are still not known.

Results: We have examined the ACP specificity of the *Streptomyces glaucescens* FabD and shown that it reacts specifically with monomeric forms of ACP, with comparable k_{cat}/K_M values for ACPs from both type II PKS and FAS systems. Incubations of tetracenomycin ACP (TcmM) with the *Escherichia coli* FAS ACP (AcpP) unexpectedly revealed that, in addition to the self-malonylation process, TcmM can catalyze the malonylation of AcpP. The k_{cat}/K_M value for the TcmM-catalyzed malonylation of *S. glaucescens* FAS ACP is two orders of magnitude smaller than that observed for the FabD-catalyzed process.

Conclusions: The ability of a PKS ACP to catalyze malonylation of a FAS ACP is a surprising finding and demonstrates for the first time that PKS ACPs and FabD can catalyze the same reaction. The differences in the catalytic efficiency of these two proteins rationalizes *in vitro* observations that FabD-independent polyketide biosynthesis proceeds only at high concentrations of a PKS ACP.

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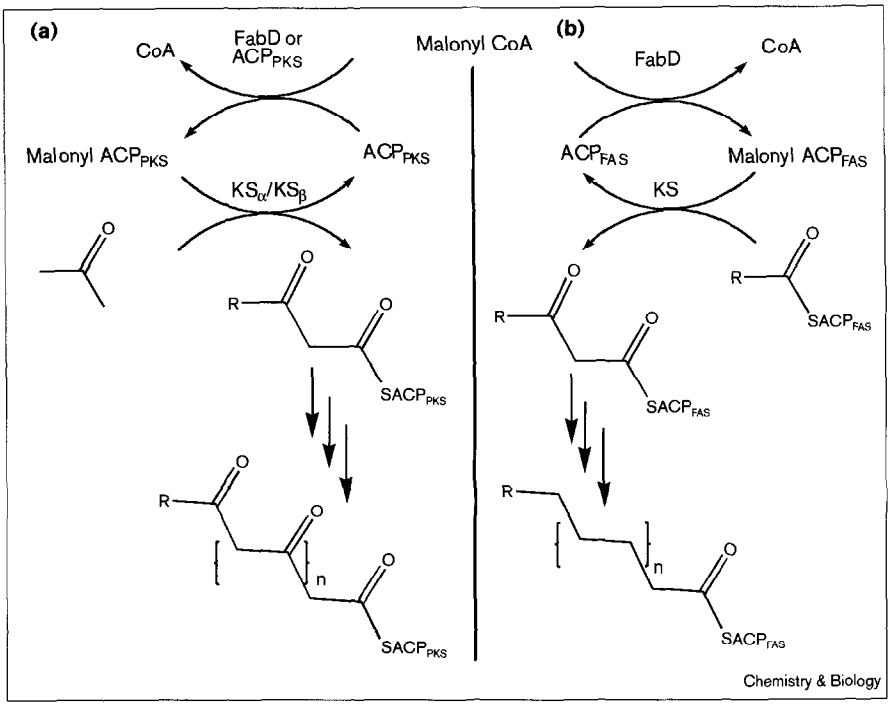
Introduction

A minimal type II polyketide synthase (PKS) in streptomycetes consists of a dissociable complex of three enzymes, an acyl carrier protein (ACP) and two subunits of the β ketoacyl ACP synthase (KSα and KSβ) [1,2]. This complex is capable of forming a polyketide by elongating an acyl ACP starter unit by multiple decarboxylative condensation steps with malonyl ACP (Figure 1). In this manner the minimal tetracenomycin (TCM) PKS produces a decaketide that is subsequently converted to TCM by the action of additional enzymes (Figure 1) [3]. The dissociable enzymes of a type II fatty acid synthase (FAS) also include an ACP and β ketoacyl ACP synthases (KSs) [4]. In this case, however, the β keto group is fully reduced by the action of three additional enzymes before the subsequent decarboxylative condensation with malonyl ACP (Figure 1). The PKS and FAS systems, therefore, use separate ACPs and KSs to generate significantly different products, but nonetheless use malonyl ACP as a substrate. The enzyme(s) responsible for *in vivo* generation of the malonyl ACP substrate for polyketide biosynthesis from malonyl CoA has generated considerable interest [3,5–7].

In the type II FAS the formation of malonyl ACP is catalyzed by a malonyl CoA:ACP acyltransferase (FabD) [5,7]. In streptomycetes and *Escherichia coli* the *fabD* gene is clustered with other fatty-acid biosynthetic genes [5,7,8]. In most type II PKS gene clusters *fabD* homologs have not been observed, suggesting that the FAS FabD might catalyze this step in polyketide biosynthesis [3,6,9]. The observation that the *Streptomyces glaucescens* FabD can catalyze the formation of malonyl ACP from malonyl CoA using both the *S. glaucescens* FAS ACP (FabC) and PKS ACP (TcmM) [3,5] is consistent with this hypothesis. Additional support for this hypothesis is that FabD has been purified from *S. coelicolor* using assays with the *S. coelicolor* PKS ACP (actI ORF3) [7]. These *in vitro* data clearly indicates a relaxed specificity for the two streptomycete FabDs (although the efficiency of the process with various ACPs has not been described). Additional support for the role of FabD in polyketide biosynthesis has been provided by *in vitro* experiments with reconstituted minimal PKS systems. Polyketide biosynthesis from malonyl CoA in a purified heterologous PKS system containing the actinorhodin KS_α (ActI ORF1) and KS_β (ActI ORF3) and frenolicin PKS ACP (FrnN) is dependent

Figure 1



Proposed role of FabD and a PKS ACP in catalyzing formation of malonyl ACP for type II **(a)** polyketide and **(b)** fatty acid biosynthetic processes. In both processes, malonyl ACP functions as a two-carbon donor in a chain elongation process to generate a product. In the polyketide biosynthetic process this product is elongated without modification of the β carbon and *in vitro* data have shown that either FabD or ACP_{PKS} can catalyze formation of malonyl ACP_{PKS} from the designated ACP_{PKS}. In fatty acid biosynthesis the elongation process occurs after the β carbon of this β ketoacyl ACP product has been fully reduced, and FabD is responsible for formation of malonyl ACP_{FAS} from the designated ACP_{FAS}.

upon addition of the *S. coelicolor* FabD [6]. The requirement of *S. glaucescens* FabD for *in vitro* polyketide biosynthesis of TCM F2 using a minimal tetracenomycin PKS and a cyclase (TcmN) has also been reported recently [3].

In seeming contrast to these observations, it has been reported that, in the absence of FabD, rigorously purified actinorhodin *holo*-ACP and other type II PKS ACPs can self-malonylate [10]. Furthermore, at high ACP concentrations it was reported that addition of equimolar concentrations of FabD decrease, rather than increase, the rate of malonyl-ACP formation. It has been argued that *in vitro* polyketide biosynthesis could proceed on a minimal PKS in the absence of FabD [11]. Such an observation has been made recently using a reconstituted minimal actinorhodin PKS. For the rate of polyketide biosynthesis in the absence of FabD to be comparable with that observed with FabD, a *holo*-ACP concentration greater than 13-fold excess of the KS_α/KS_β constituents and 20-fold greater than the FabD concentration used in the control experiment, is required [11].

In an attempt to unravel these seemingly contradictory observations we undertook a more detailed investigation of the ACP specificity of the *S. glaucescens* FabD. This work has clearly demonstrated that for FabD the k_{cat}/K_m for a PKS ACP and that observed with either the *E. coli* ACP (AcpP) or *S. glaucescens* FAS ACP (FabC) are comparable. At high concentrations the PKS ACP dimerizes and is no longer a substrate for FabD. These observations provide a resolution of the anomalous results regarding the ability of streptomycete FabD to malonylate PKS ACPs. We have also shown that in addition to self-malonylation the *holo*-form of a PKS ACP can catalyze the malonylation of the *E. coli* AcpP and the *S. glaucescens* FAS ACP, albeit at a less efficient level than that observed with FabD. These observations rationalize the observed differences in concentration of FabD and PKS *holo*-ACP required for catalysis of malonyl ACP formation during *in vitro* polyketide biosynthesis with reconstituted PKS systems.

Results

Expression, purification and modification of the substrates for the FabD assay

To determine the substrate specificity of the *S. glaucescens* FabD a number of substrates were purified, including FabC (*S. glaucescens* FAS ACP), TcmM and FrnN (frenolicin PKS ACP). As previously reported the type II PKS ACPs (but not FAS ACPs) were expressed in *E. coli* predominantly in the *apo*-form [12]. A high-performance liquid chromatography (HPLC) assay was used to confirm 95% or greater *in vitro* conversion of these *apo*-ACPs to the corresponding *holo*-ACP using *holo*-ACP synthase (ACPS) [12]. Further purification of FrnN and TcmM was carried out using gel-filtration chromatography using a Superdex 75 column. Under these conditions, a multimeric form of the ACPs, which predominated despite the presence of dithiothreitol (DTT), was resolved from the monomeric form. Addition of 1% Triton to the buffer led to an increase in the monomeric form of FrnN. Prolonged treatment with

Table 1

Kinetic parameters for ACPs as substrates for malonylation by FabD and TcmM from *S. glaucescens*.

Catalyst	Substrates	k_{cat} (min^{-1})	K_m (μM)	k_{cat}/K_m ($\mu M^{-1}min^{-1}$)
FabD	AcpP from *E. coli*	1.2	42 ± 16	0.03
	FabC from *S. glaucescens*	1.4	87 ± 14	0.02
	FrenN (monomer)	1	33 ± 19	0.01
TcmM	AcpP from *E. coli*	1.7×10^{-2}	22 ± 7	7.7×10^{-4}

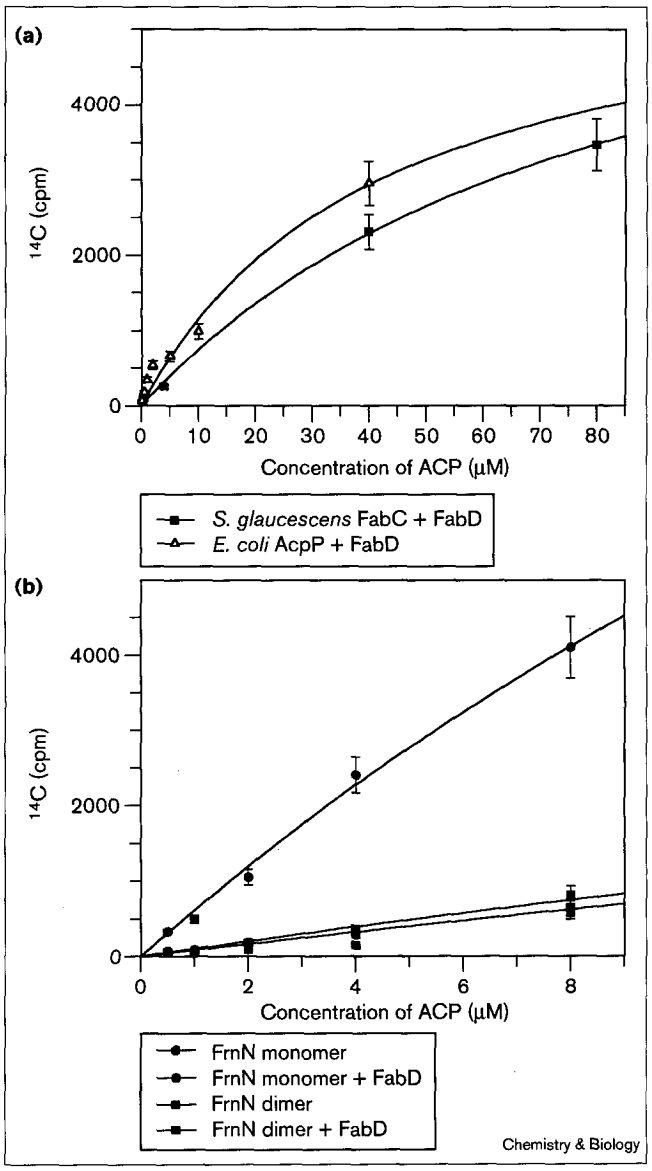
DTT or addition of Triton, however, did not lead to increased amounts of the monomeric form of TcmM. This multimeric form of TcmM and FrnN appeared to be a dimer with a molecular mass between 12,000 Da and 29,000 Da. Dimeric forms of PKS ACPs have previously been observed using both electron spray mass spectrometry (ES-MS) and native polyacrylamide gel electrophoresis (PAGE) analyses [11,13].

Substrate specificity of FabD

To investigate substrate specificity of the *S. glaucescens* FabD, the relative apparent K_m and k_{cat} values were determined for two FAS ACPs and the monomeric form of FrnN (Table 1). To ensure that presence of any contaminating FabD from *E. coli* did not affect kinetics studies, all purified substrates were pre-incubated with 2 mM phenylmethylsulfonyl fluoride (PMSF) as previously described [10]. No substantial difference was observed in the reactivity of FabD with either FAS ACP substrate (Table 1; Figure 2a). In the case of FrnN, rates of self-malonylation were compared with FabD-catalyzed malonylation using simultaneous assays in the presence and absence of FabD. At low concentrations of FrnN the rate of malonylation was substantially faster in the presence of FabD (Figure 2b). At high FrnN concentrations, however, the rates of the two processes were indistinguishable (data not shown). Using data obtained at the lower FrnN concentrations and adjusting for the amount of product formed via self-malonylation allowed an estimation of the k_{cat} and K_m values for FabD-catalyzed malonylation of FrnN (Table 1). These kinetic parameters were remarkably similar to those obtained with the FAS ACPs (*S. glaucescens* FabC and the *E. coli* AcpP). The results of kinetic analyses that were conducted with the monomeric form of TcmM were indistinguishable from those conducted with FrnN suggesting that this PKS ACP reacted with the same efficiency with FabD. Difficulties associated with obtaining sufficient quantities of the monomeric form of TcmM to carry out kinetic analyses in triplicate, however, prevented a determination of the kinetic parameters with this substrate.

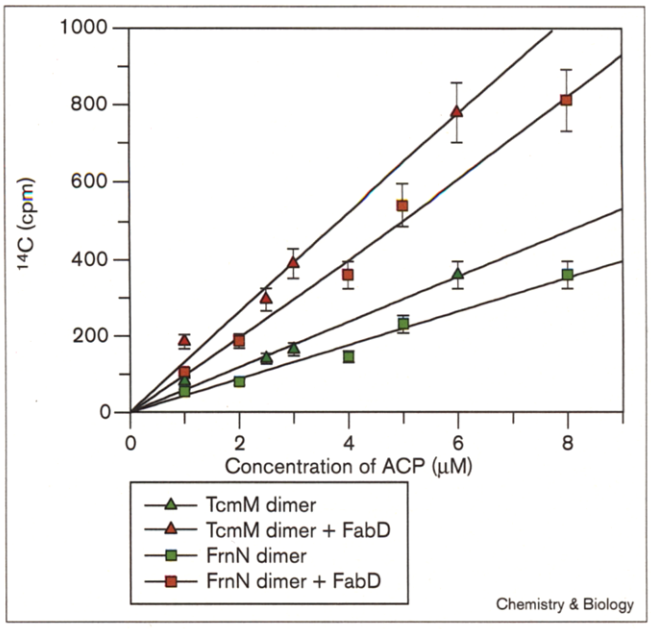
The FrnN and TcmM dimers, in contrast to the monomeric ACPs, were poor substrates for FabD. The rates of malonylation of FrnN dimer by FabD were only marginally higher than the rates of self-malonylation (Figures 2b,3) making it difficult to estimate the k_{cat} and K_m values. A similar observation was made with the TcmM dimer (Figure 3). Interestingly, the rate of self-malonylation of the FrnN monomer and dimer were indistinguishable, indicating that thiol group of both the dimer and monomer forms was equally accessible (Figure 2b). The rates of self-malonylation of the dimeric forms of

Figure 2



Kinetic analysis of the substrate specificity of FabD for **(a)** FabC (*S. glaucescens*) and AcpP (*E. coli*) and **(b)** FrnN. Assays in the presence of FabD were *S. glaucescens* FabC (blue squares), *E. coli* AcpP (yellow triangles), FrnN monomer (green circles), and FrnN dimer (green squares). Assays carried out in the absence of FabD were FrnN dimer (red squares), and FrnN monomer (red circles). The rates of self-malonylation of FrnN monomer and dimer are indistinguishable.

Figure 3



Comparison of the rate of malonyl ACP formation for dimeric forms of TcmM (green triangles) with FrnN (green squares) in the absence of FabD. Assays in the presence of FabD are also shown (TcmM, red triangles; FrnN, red squares).

TcmM and FrnN were also indistinguishable (Figure 3). The *S. glaucescens* FabD exhibited saturation kinetics in response to increasing concentrations of malonyl CoA with an apparent K_m of 50 ± 2 μM.

Malonylation of the *E. coli* AcpP by TcmM

An incubation of a mixture of AcpP and TcmM with [2-^{14}C]malonyl CoA under standard conditions gave significantly more radioactive malonyl ACP than assays conducted with AcpP or TcmM alone. As each ACP was individually pretreated with 2 mM PMSF this result suggested that the increase is a result of the catalytic activity of TcmM rather than an impurity of FabD. Similar observations were made using FrnN, but not the *apo* form of TcmM, in place of TcmM. A series of experiments was carried out to investigate this surprising phenomenon.

In one set of analyses the rate of malonylation under varying concentrations of TcmM and a constant concentration of AcpP was determined. Under all TcmM concentrations, addition of the *E. coli* AcpP led to dramatic increases in the amount of malonyl ACP formed and a linear relationship was observed between the concentration of TcmM and the rate of malonyl ACP formation (Figure 4a). In a second set of experiments the TcmM concentrations were constant (1 μM) and the *E. coli* AcpP concentrations were varied (Figure 4b). Under these conditions the amount of radioactive malonyl ACP formed in the assays also increased dramatically in the presence of *E. coli* AcpP. In this case, however, typical saturation kinetics were observed with increasing concentrations of AcpP. Similar saturation kinetics were observed using the *S. glaucescens* FabC (data not shown). These results are consistent with TcmM catalyzing the malonylation of FabC and allowed the apparent K_m and k_{cat} values for this process with the *E. coli* AcpP to be determined (Table 1). Comparatively, k_{cat} for malonylation of FabC by TcmM is less than 2% of that observed with FabD. The K_m of TcmM and FabD for the *E. coli* FAS ACP, however, were remarkably similar.

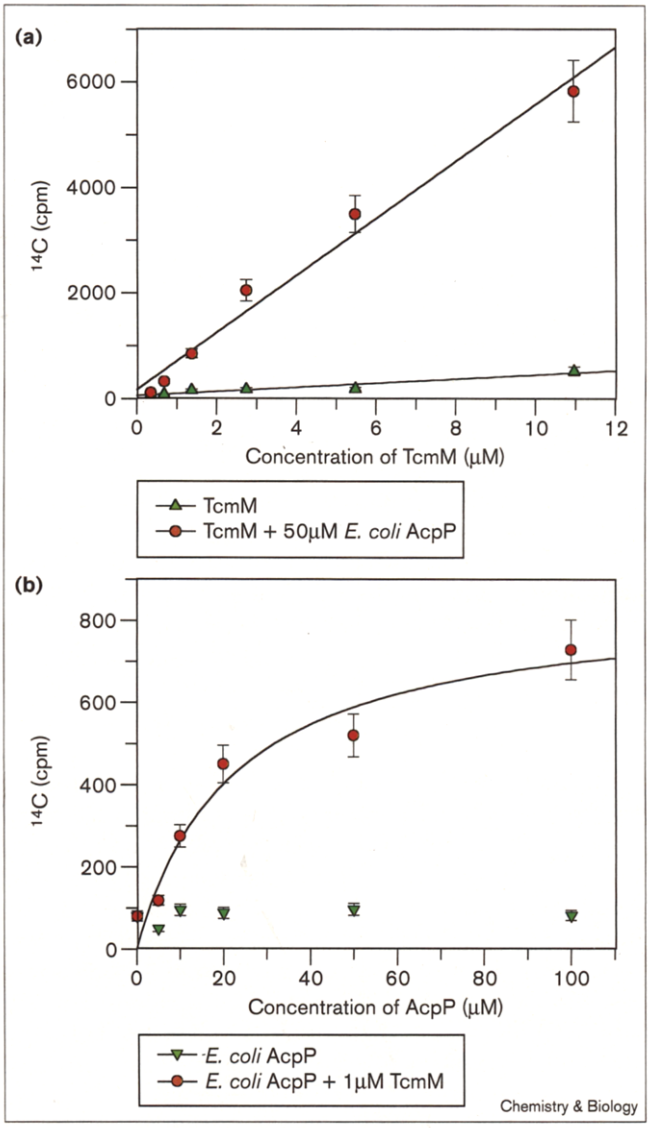
Additional evidence that the *E. coli* AcpP was malonylated in the presence of the His-tagged TcmM was accomplished by Ni-chelate column chromatography. An assay mixture containing labeled malonyl CoA, FabC and His-tagged TcmM was incubated for 15 minutes and subsequently loaded onto a Ni-chelate column. Most of the radioactive protein that could be eluted from the resin did so without addition of imidazole, consistent with the production of ^{14}C-labeled malonylated FabC. A quantitatively similar result was obtained in a control experiment when FabC was incubated with FabD and [2-^{14}C]malonyl CoA. In another control experiment when radiolabeled malonylated His-tagged TcmM was generated, the majority of radioactive material that could be eluted from the column did so in the presence of 500 mM imidazole.

Finally, autoradiography was used to confirm the formation of malonylated AcpP in the presence of TcmM (Figure 5). The same-sized labeled product was observed from sodium dodecyl sulfate (SDS)–PAGE analyses of incubations of FabC and [2-^{14}C]malonyl CoA carried out in the presence of either FabD or PMSF-treated TcmM. No detectable products were observed when PMSF-treated TcmM and FabC were incubated individually with [2-^{14}C]malonyl CoA under the same conditions (Figure 5).

Discussion

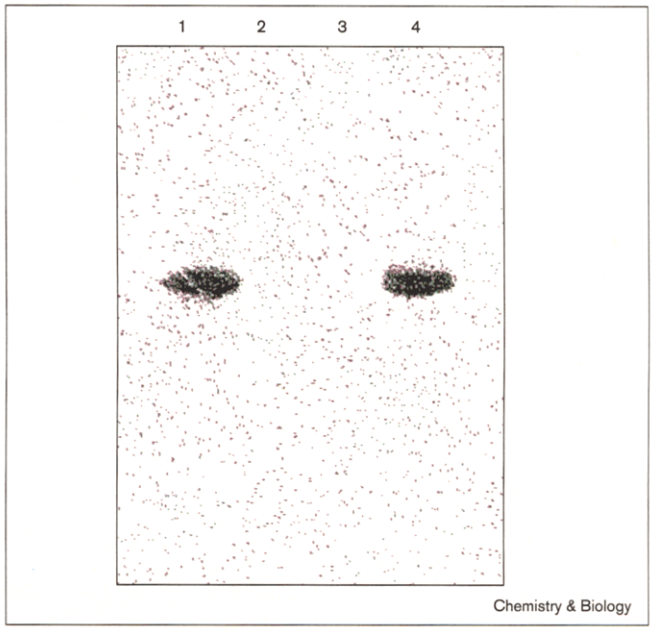
Results from previous analyses of FabD of *S. coelicolor* and *S. glaucescens* have clearly indicated that these enzymes are capable of processing FAS ACPs and PKS ACPs [3,5–7,11]. Furthermore, it has been shown that under certain conditions FabD is required for *in vitro* polyketide biosynthesis with a reconstituted minimal polyketide synthase. As FabC is the most abundant ACP in *S. glaucesens* [5], the *S. glaucesens* FabD would presumably need to process TcmM and FabC similarly if it were to play a role in catalyzing polyketide biosynthesis *in vivo*. This hypothesis is supported by the kinetic analyses carried out in this study. It is also interesting that only the monomeric form of either TcmM or FrnN were effective substrates for FabD. The same requirement for the monomeric form of the actinorhodin ACP (actI ORF3) has recently been

Figure 4



Kinetic analysis of the reaction malonylation of the *E. coli* AcpP by TcmM as a function of **(a)** TcmM and **(b)** AcpP. (a) Assays were performed in the presence of malonyl CoA (50 μM) and AcpP (50 μM) and varying concentrations of TcmM (red circles). A simultaneous set of assays without AcpP was conducted (green triangles). (b) Assays were performed in the presence of malonyl CoA (50 μM) and TcmM (1 μM) and varying concentrations of AcpP (green triangles). A simultaneous set of assays without TcmM was also conducted (red circles).

Figure 5



Autoradiography of 15% SDS–PAGE gel showing *E. coli* FabD and TcmM malonylation of AcpP with ^{14}C-labeled malonyl CoA: lane 1, AcpP (50 μM) + TcmM (8 μM); lane 2, TcmM (8 μM) only; lane 3, AcpP (50 μM) only; lane 4, AcpP (50 μM) + FabD (0.1 μM).

demonstrated for *in vitro* polyketide biosynthesis with the minimal actinorhodin polyketide synthase [11].

At low concentrations of TcmM and FrnN the rate of malonyl ACP formation in the presence of FabD differs significantly from the rate of self-malonylation. At higher concentrations of ACP (above 30 μM) no significant differences in the rates of these two processes were observed (data not shown). This latter observation is similar to a recent report that the rate of malonyl ACP formation by high concentrations of the actinorhodin ACP (50 μM) is not enhanced by equimolar concentrations of the *S. coelicolor* FabD [10]. These observations are partly because of ACP concentration exhibiting a linear relationship with the rates of self-malonylation, and saturation kinetics with the rates of FabD-catalyzed malonylation. Thus, the greatest difference between these two processes is observed at low ACP concentrations. The reason that FabD produces no enhancement of malonylation at higher ACP concentrations is probably that ACP is able to dimerize under such conditions. In this study it has been demonstrated that the TcmM and FrnN dimers can self-malonylate at the same rate as the monomeric forms, but are no longer efficient substrates for FabD.

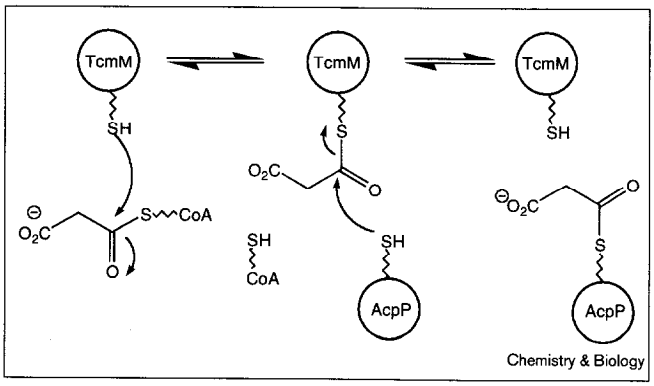
Although FabD is able to catalyze malonyl ACP formation with TcmM and FrnN, the results in this current study also demonstrate clearly that both of these ACPs are able to catalyze self-malonylation. This unusual activity, which has been previously reported for the actinorhodin and griseusin ACPs, thus appears to be a general phenomenon with type II PKS ACPs. It has previously been suggested that self-malonylation of actinorhodin ACP might derive from residual ACPS or contaminating *E. coli* FabD that is present in the *E. coli* BL21 DE(3) extracts used to obtain the *apo*-ACP [6]. In the current study SDS–PAGE analysis revealed no detectable levels of FabD and ACPS in the

ACP samples after purification by gel filtration. Furthermore, PMSF pretreatment of the ACPs did not affect the rate of self-malonylation, but completely inhibited the FabD-catalyzed malonylation of the *E. coli* AcpP in a control assay. Finally the observed rate of ACP self-malonylation in a 30 second assay could not be accounted for by trace levels of ACPS.

Surprisingly, the rate of self-malonylation of TcmM and FrnN in dimeric form was indistinguishable from that observed with the monomeric form suggesting that in both forms the 4′-phosphopantetheine arm are equally accessible. The possibility that the two forms exist in rapid equilibrium is discounted by the observation that under the same assay conditions FabD reacts only with the monomer. The dimeric forms of TcmM and FrnN are, therefore, unlikely to be a result of a disulfide bond formation between the two 4′-phosphopantheine arms. Consistent with this conclusion are the observations that the *apo* form of FrnN, which is purified by gel filtration, exists predominantly in dimeric form and that prolonged inhibition of *holo*-TcmM dimer with DTT did not lead to significant formation of the TcmM monomer. As FrnN contains no cysteine residues it clear that the dimeric form of FrnN is also not a result of a cysteine disulfide bond. The observation that the dimeric forms of TcmM and FrnN do not involve either kind of disulfide bond is surprising because disulfide dimers have been previously reported for some PKS ACPs [10,13].

In addition to catalyzing self-malonylation, the monomeric and dimeric forms TcmM and FrnN can catalyze the malonylation of the *E. coli* AcpP and the *S. glaucescens* FabC. Presumably in this process the malonyl group is first transferred from malonyl CoA onto the 4′-phosphopantetheine arm of the PKS ACP (Figure 6). The malonyl group is subsequently transferred to the 4′-phosphopantetheine arm of AcpP (it has been demonstrated previously that self-malonylation process with the actinorhodin ACP is reversible, indicating transfer of malonyl group from malonyl ACP to the 4′-phosphopantetheine of CoA). Consistent with this hypothesis is the observation that the *apo* form of TcmM is unable to catalyze the malonylation of AcpP. The mechanism by which a PKS ACP catalyzes AcpP malonylation, however, remains to be determined.

Figure 6



Proposed mechanism for TcmM-catalyzed malonylation of FabC.

In this study, AcpP malonylation has been shown to be more efficiently catalyzed by FabD than by TcmM. The kinetic studies were carried out with 50 μM malonyl CoA, below the reported K_m (219 μM) for the self-malonylation of the actinorhodin ACP. At higher concentrations of malonyl CoA the difference in the k_{cat} for the FabD and PKS ACP-catalyzed processes might be less dramatic. Nonetheless, it is clear that FabD is a more efficient catalyst for malonylation and it is not surprising that high concentrations of a PKS ACP (20 μM) are required for FabD-independent polyketide biosynthesis to occur *in vitro* at the same rate as that observed with 1 μM of FabD. The reason why the PKS ACP needs to be in excess to the other components of the reconstituted PKS in order to support FabD-independent polyketide biosynthesis is unclear, but might reflect a diminished ability to self-malonylate while associated with the KS_α and KS_β.

Significance

The malonyl acyl carrier protein (ACP) is required for both fatty acid and polyketide biosynthesis presumably using FabC (fatty acid synthase ACP) and TcmM (the tetracenomycin ACP), respectively. *Streptomyces glaucescens* contains two different catalysts, FabD (malonyl CoA:ACP acyltransferase) and TcmM, both of which can catalyze *in vitro* malonylation of FabC and TcmM. Expression of the *tcmM* gene is not detected until 18 hours of growth of *S. glaucescens* [14] and attempts to obtain a *S. glaucescens fabD* mutant have been unsuccessful [2]. The malonylation of FabC by FabD, and not by TcmM, is therefore undoubtedly the *in vivo* process used for fatty acid biosynthesis. The *in vivo* process primarily responsible for the malonylation of TcmM for polyketide biosynthesis, however, is less clear.

The *S. glaucescens* FabD is able to malonylate fatty acid synthase (FAS) and polyketide synthase (PKS) ACPs with similar efficiency, and has been shown previously, along with the *S. coelicolor* FabD, to be required for *in vitro* polyketide biosynthesis with a reconstituted polyketide system [3,6]. These observations indicate that as long as FabD is available *in vitro*, it would play a role catalyzing malonylation of TcmM for polyketide biosynthesis. Thus, as previously suggested, FabD represents a link between the processes of polyketide and fatty acid biosynthesis [5,7]. It has recently been shown, however, that in *Escherichia coli* FabD is associated with β-ketoacyl ACP synthase isozymes of the FAS [15]. Furthermore, recent analyses of *S. glaucescens* have indicated

that some of the FAS enzymes are associated *in vivo* (N.A. Smirnova and K.A.R., unpublished observations). The availability of FabD for catalysis of TcmM malonylation, therefore, remains to be determined.

In this study it has been shown that TcmM catalyzes both self-malonylation and malonylation of other ACPs, but is a relatively poor catalyst when compared with FabD. Nevertheless, the actinorhodin ACP (actI ORF3) has been shown previously to support FabD-independent polyketide biosynthesis *in vitro* when present in substantial molar excess to the other PKS components. Presumably FabD-independent polyketide biosynthesis could also occur *in vivo* if a similar stoichiometry of PKS components were present. Indeed, additional copies of *tcmM* have been previously reported to lead to increased levels of tetracenomycin C [16]. The stoichiometry of the minimal PKS components when only one copy of each respective gene is present is unknown. Under such conditions it is unclear if TcmM self-malonylation is physiologically significant.

Materials and methods

Expression and purification of FabC (S. glaucescens) *FrnN, TcmM and* holo-*ACPS* (E. coli)

The *S. glaucescens fabC* was PCR amplified from pWHM194 (kindly provided by C. Richard Hutchinson, University of Wisconsin). The DNA PCR primers were 5′-GAAAACGAAGGAGCGGCCC*ATATG*GCCG-GCCA-3′ (rightward primer mapping on upstream sequence of *fabC* and having *Nde*I site (italicized) introduced to facilitate cloning) and 5′-GGCAGGCAACGAGCC*TCGAG*GATCAGGCT-3′ (leftward primer mapping on downstream sequence from *fabC* and having a *Xho*I site (italicized) introduced to facilitate cloning). The *Nde*I–*Xho*I digested PCR product was cloned into expression vector pET 30a(+) to give pGF100. *E. coli* BL21(DE3)pLysS/pGF100 transformants were used for expression of FabC with a carboxy-terminal His-Tag.

The expression plasmids containing genes for *Streptomyces* ACPs and *holo*-ACPS were kindly provided by C.T. Walsh, Harvard Medical School. The *Nde*I–*Hind*III digested *dpj* gene, encoding ACPS, was cloned into pET30a(+) to give pGF101. *E. coli* BL21(DE3)pLysS/pGF101 transformants carrying pGF101 were used for expression of ACPS with a carboxy-terminal His-Tag.

Transformed cells were grown at 37°C to an optical density 0.4–0.6, induced with isopropyl-β-D-thiogalactopyranoside (IPTG) and incubated for an additional 3–5 h [17]. The ACPs were partially purified by ammonium sulfate precipitation as described previously [5] and further purified by gel filtration using a Superdex-75 column. This step also allowed the dimeric and monomer forms of FrnN and TcmM were resolved. The His-tag TcmM was additionally purified using nickel-chromatography according to manufacture protocols (Novagen). The *holo*-ACPS was similarly purified by nickel-chromatography. Purity of the various ACPs was evaluated using SDS–PAGE.

Expression and purification of FabD

The *S. glaucescens* FabD was expressed and purified from *E. coli* as described previously [18].

Modification of apo-*ACPs to* holo-*ACP*

In a final volume of 1 ml, 35 μM substrate (*apo*-FrnN or TcmM) was incubated at 37°C with 1 mM CoA, 50 mM Tris-HCl, 20 mM $MgCl_2$, 1 mM DTT, 2 mM EDTA, and 0.25 μM purified ACPS for 4–8 h at pH 8.8. A previously described 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 [12].

PMSF inhibition of FabD

Purified TcmM, FrnN and FabC (*S. glaucescens*) and AcpP (*E. coli*, Sigma Chemical Company) were treated with PMSF prior to incubation with malonyl CoA as described previously [10].

FabD assay

The FabD assay was carried out as described previously [18]. Briefly, a reaction mixture containing 50 μM malonyl-CoA (5 μM [2-^{14}C]malonyl CoA + 45 μM cold malonyl CoA) (50,000 cpm), 1 μM DTT, 2 μM EDTA, 50 mM phosphate buffer and 0.1 mM FabD in a final volume 100 μl was incubated for 30 s at 37°C at pH 7.0 and subsequently quenched by addition of 200 μl of an ice-cold trichloroacetic acid (TCA) solution (10%). Precipitation was completed by incubation on ice for 10 min. Centrifigation yielded a pellet that was washed with 10% ice-cold TCA and resuspended in 2% SDS, 20 mM NaOH solution. The suspension was combined with 5 ml of a scintillation cocktail and analyzed with a scintillation counter.

Kinetic determinations

The apparent K_m values of FabD for FabC (*S. glaucescens*), AcpP (*E. coli*) and FrnN was determined under standard conditions using 50 μM malonyl-CoA (5 μM [2-^{14}C]malonyl CoA + 45 μM malonyl CoA) (50,000 cpm) and variable concentrations of the ACP substrates (all ACPs were treated with PMSF prior the reaction). To account for any loss in FabD activity or 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. In the case of TcmM and FrnN, simultaneous assays were also carried out in the absence of FabD, allowing the process of self-malonylation to be followed.

The K_m value of FabD for malonyl CoA was determined using 20 μM of the *E. coli* AcpP (Sigma) and variable concentrations of [2-^{14}C]malonyl CoA. All reactions and controls were performed at least in triplicate. All kinetic values were obtained by nonlinear regression analysis using GraFit 4.0.

Malonylation of E. coli *AcpP by TcmM*

The K_m and relative k_{cat} value for malonylation the AcpP of *E. coli* by TcmM was determined under standard conditions (TcmM (1 μM), 50 μM malonyl-CoA (50 μM [2-^{14}C]malonyl CoA + 45mM cold malonyl CoA) and variable concentrations of AcpP). A second set of analyses in which the AcpP (50 μM) was held constant and the TcmM concentration was varied was also conducted. In all experiments the TcmM and AcpP were treated with PMSF prior the reaction.

Autoradiography of ^{14}C–labeled malonylated AcpP

Reaction mixtures containing 8 μM [2–^{14}C]malonylCoA and 20 μM of the *E. coli* AcpP were incubated with either 8 μM TcmM or 0.1 μM FabD at 37°C for 15 min. Two control incubations with 8 μM [2–^{14}C]malonyl CoA and either AcpP (20 μM) or TcmM (8 μM) were carried out simultaneously. All incubations quenched with 100% ethanol and loaded onto 15% SDS–PAGE gel. Dried, stained gels were analyzed by autoradiography.

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