Dissecting the Component Reactions Catalyzed by the Actinorhodin Minimal Polyketide Synthase[†]

Pedro Beltran-Alvarez, Russell J. Cox,* John Crosby, and Thomas J. Simpson

School of Chemistry, University of Bristol, Cantock's Close, Bristol, BS8 1TS, UK

Received August 31, 2007

ABSTRACT: The actinorhodin (act) minimal polyketide synthase (PKS) from *Streptomyces coelicolor* consists of three proteins: an acyl carrier protein (ACP) and two β-ketoacyl ACP synthase components known as KS_α and KS_β. The act minimal PKS catalyzes at least 18 separate reactions which can be divided into loading, initiation, extension, and cyclization and release phases. Two quantitative kinetic assays were developed and used to measure individual rate and Michaelis constants for loading, initiation and extension steps. In the minimal PKS, the reaction between malonyl CoA and ACP to form malonyl ACP (loading) is the rate-limiting step ($k_{cat} = 0.49$ min^{-1}, $K_M = 207$ μM). This reaction increases 5-fold in rate in the presence of $KS_\alpha KS_\beta$ ($k_{cat} = 2.3$ min^{-1}, $K_M = 215$ μM). In the presence of *S. coelicolor* malonyl CoA: ACP transacylase (MCAT), the rate of loading increases and the kinetic parameters of malonyl-ACP as a substrate of $KS_\alpha KS_\beta$ can be measured ($k_{cat} = 20.6$ min^{-1}, $K_M = 2.4$ μM). Under these conditions, it appears that decarboxylation of malonyl-ACP to form acetyl-ACP (initiation) is the rate-limiting step. When an excess of acetyl ACP is supplied, either chain extension, cyclization, or release steps become rate limiting ($k \approx 60$ min^{-1}). No ACP-bound intermediates could be observed, suggesting that partially or fully extended chains do not accumulate because chain extension is rate limiting under these conditions and that cyclization and release are fast. *apo*-ACP acts as a mixed inhibitor of malonyl ACP binding to KS_α/KS_β ($K_{ic} = 50$ μM, $K_{iu} = 137$ μM), but *apo*-ACP does not appear to inhibit MCAT.

The polyketide actinorhodin **1** (act) is produced by the actinomycete *Streptomyces coelicolor* A(3)2. Hopwood and co-workers first linked the biosynthesis of **1** to a Type II polyketide synthase (PKS[1]) gene cluster in 1984 (*1*, *2*). Pioneering experiments led to the development of a mutant strain of *S. coelicolor* (CH999) in which the act cluster had been deleted. This was used as a host for plasmids containing subsets of the act genes. From these studies it was found that *act*I ORFs 1−3 were the minimal set of genes required to produce polyketide products: SEK4 **2** and SEK4b **3** *in vivo* (*3*, *4*). The *act*I ORF1 and *act*I ORF2 genes encode two β-ketoacylthiolester synthase components, known as KS_α and KS_β respectively, each of *ca.* 45 kDa, while *act*I ORF3 encodes an acyl carrier protein (ACP) of 9441 Da. The minimal PKS condenses eight malonate units (as in actinorhodin) to form an octaketide chain. The minimal PKS also catalyzes the initial cyclizations to form SEK4 **2,** in which the cyclization corresponds to that found in actinorhodin **1**, and SEK4b **3** with an aberrant mode of cyclization. The *in vivo* work was followed by *in vitro* studies of the minimal PKS using purified proteins (*5*−*7*) in which **2** and **3** are also produced.

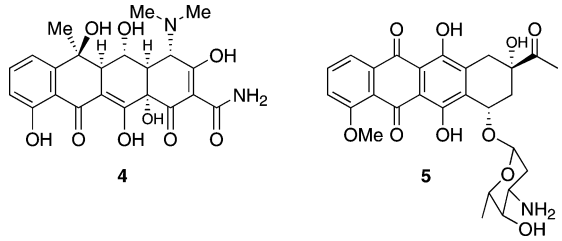


FIGURE 1: Oxytetracycline **4** and daunorubicin **5**.

The act PKS has proven to be a very useful model for understanding other Type II polyketide synthases such as those involved in antibiotic (e.g., oxytetracycline **4**) and anticancer compound (e.g., daunorubicin **5**) biosynthesis in other Streptomycetes (Figure 1) (*8*, *9*). The act PKS is iterative; that is, the single set of proteins acts several times to produce a single final product. Because of this, the act PKS is also a very useful model for the iterative Type I PKS found in fungi for example (*10*, *11*, *12*, *13*), and the individual reactions catalyzed by the act PKS are the same as those catalyzed by the chain extension domains of single modules of bacterial Type I modular PKS (*14*). Thus, understanding of the act PKS serves to underpin advances in understanding the other main classes of PKS proteins in bacteria and fungi.

The act minimal PKS catalyzes a number of individual reactions (Scheme 1). First, malonate must be transferred

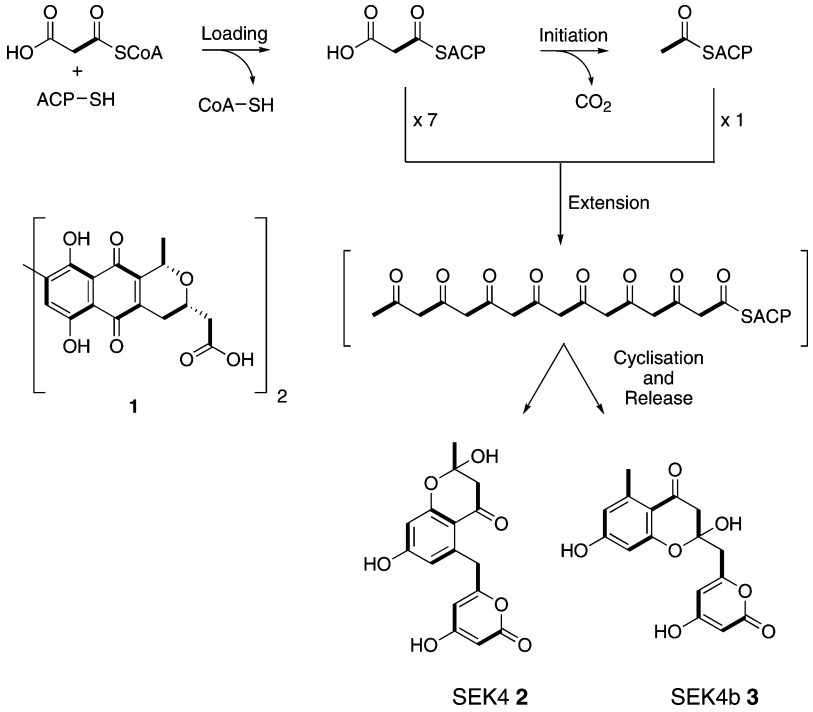
[†] We thank the European Union FP6 MC-EST project BRISENZ for funding, EC contract no: QLK3-CT-2002-01940.

* Author to whom correspondence should be addressed. Tel: +44 (0) 117 928 9184. Fax: +44 (0) 117 929 8611. E-mail: r.j.cox@bris.ac.uk.

[1] Abbreviations: PKS, polyketide synthase; act, actinorhodin; ACP, acyl carrier protein; PP, phosphopantetheine; MCAT, malonyl CoA: ACP transacylase; DTT, dithiothreitol, TCEP, tris(2-carboxyethyl)-phosphine; αKG, α-ketoglutaric acid; KDH, α-ketoglutarate dehydrogenase; KS, β-ketoacyl-ACP synthase; TPP, thiamine pyrophosphate; NAD^+/NADH, nicotinamide adenine dinucleotide (oxidized/reduced); Tcm, tetracenomycin; Sch, *S. halstedii* spore pigment; Pms, pradimycin; Fren, frenolicin; Zhu, R1128; Gra, granaticin; WhiE, white E; RLS, rate limiting step.

10.1021/bi701784c CCC: $37.00
Published on Web 11/23/2007

Scheme 1: Biosynthesis of Octaketides by the Actinorhodin Minimal PKS



from CoA to *holo*-ACP. This loading reaction can be catalyzed by a malonyl transferase enzyme known as MCAT (*6*, *15*, *16*), but it can also be self-catalyzed (*17*−*19*). Second, malonyl ACP must be decarboxylated, in an initiation step, to form acetyl ACP which forms the starter unit for polyketide biosynthesis. KS_β has been shown to catalyze this reaction (*20*). Third, the chain is extended to 16 carbon atoms, normally by seven extension reactions, using malonyl ACP, catalyzed by iterative cycles of KS_α (*5*, *21*). Finally, the fully formed chain is cyclized and released from the synthase. Thus, the act minimal PKS catalyzes at least 18 separate reactions which can be classified into loading, initiation, extension, and cyclization and release steps.

Remarkably, little is known of the absolute or relative rates of the component reactions of the act minimal PKS. For example, although the overall transformation from malonyl CoA to octaketides has been studied by Khosla and coworkers (*22*−*24*) and by us (*7*, *21*), the rate-limiting step (RLS) of the act PKS is unknown. These studies have employed different assays. For example a thin layer chromatography (TLC) method was used by Khosla and coworkers to measure radioactive octaketide production (*22*−*24*), whereas we used an HPLC method for the detection of octaketides **2** and **3** in minimal PKS assays (*7*, *21*). These discontinuous assays require multiple measurements and make the gathering of precise kinetic data for PKS complexes cumbersome. We have also described the use of electrospray mass spectrometry (ESMS) to monitor the extent of transfer of phosphopantetheine (PP) groups to *apo*-ACP (*18*), the transfer of acyl groups onto *holo*-ACP (*25*), the transfer of acyl groups between ACP species (*26*), and the decarboxylation of malonyl-ACP to acetyl-ACP (*20*), but ESMS is unsuited to precise kinetic measurements required for a detailed study of the kinetics of the minimal PKS.

We now report a kinetic dissection of the act minimal PKS in which discrete rate and Michaelis constants for the individual loading, initiation, and extension reactions are ascertained. This has involved the development of two continuous quantitative assays for the act PKS which can be used to probe the rates of individual catalytic steps under a variety of experimental conditions.

EXPERIMENTAL SECTION

Materials. Coenzyme A (CoA), malonyl CoA, α-ketoglutaric acid (αKG), α-ketoglutarate dehydrogenase (KDH), and thiamine pyrophosphate (TPP) were purchased from Sigma and NAD^+ was obtained from Fluka. Dithiothreitol (DTT) and tris(carboxyethyl)phosphine (TCEP) were obtained from Pierce.

Expression and Purification of Proteins. The expression and purification of act *holo*-ACP, his_6-$KS_\alpha KS_\beta$, and *S. coelicolor* his_6-MCAT have been described previously (*7*, *16*). ACP was heterologously expressed in *E. coli* BL21 and purified by fractional precipitation with ammonium sulfate and then by anion chromatography and gel filtration (*25*, *27*). His_6-MCAT was expressed in *E. coli* BL21 and purified by Ni-affinity chromatography and then by anion chromatography (*16*). His_6-$KS_\alpha KS_\beta$ was expressed in *S. coelicolor* CH999 and purified by Ni-affinity chromatography (*7*).

ESMS of ACP Species. ESMS analysis was performed on a QSTAR XL system (Applied Biosystems, Foster City, CA) equipped with a chip-based NanoESI source, Nanomate 100 (Advion Biosciences, Ithaca, NY) using Chipsoft software (version 5.1). The Nanomate 100 source was operated using a 400 nozzle chip, with a delivery pressure of 0.3 psi and spray voltage of 1.40 kV. The QSTAR XL was controlled using Analyst QS software (version 1.1) in positive TOF MS mode. All samples were subjected to the desalting and concentration procedure of Winston before analysis (*28*).

α-Ketoglutarate Dehydrogenase Assays (KDH assay). The study of self-malonylation kinetics by the α-ketoglutarate

dehydrogenase (KDH) complex was performed by measuring the change in A_{340} read by a SpectraMax 190 Plate Reader, from Molecular Devices. All enzymatic reactions were performed in a final volume of 200 μL in 96-well microplates (half-area, UV-transparent plates, Corning), in triplicate. Final concentrations of each component in the assay were as follows: 100 mM phosphate buffer (pH 7.3), 1 mM EDTA, 1 mM TCEP, 2 mM α-ketoglutaric acid (αKG), 0.5 mM NAD^+, 0.4 mM thiamine pyrophosphate (TPP), 1 mM $MgCl_2$ and 80 mU KDH. A working stock of 10 times their final concentrations of EDTA, TCEP, αKG, and NAD^+ was made up. Assays were started by addition of *holo*-ACP and initial rates measured from the absorbance traces during the first 30 to 60 s (7 to 15 time points).

Spectrophotometric Assays of act *Minimal PKS ($KS_\alpha KS_\beta$ assay).* All enzymatic reactions were performed in a final volume of 200 μL using an absorbance plate reader as for the KDH assay. All the assays were performed in 100 mM phosphate buffer (pH = 7.3) at 30 °C, in triplicate. Incubation of *holo*-ACP with TCEP (1 mM final concentration from 100 mM stock, freshly prepared) for 60 min at 30 °C provided the monomerized, active *holo*-ACP. $KS_\alpha KS_\beta$ was incubated for 2 min in the reaction mixture containing malonyl CoA, EDTA, and TCEP, which was pre-equilibrated at 30 °C in the microplate reader. Final concentrations of all components were 100 mM phosphate buffer, 2 mM EDTA, 1 mM TCEP, 10 to 1000 μM malonyl CoA, 0.05−5 μM $KS_\alpha KS_\beta$, and 0.1−160 μM *holo*-ACP. *S. coelicolor* MCAT (0.5−2.5 μM, final concentration) was added to the reaction mixture to measure the kinetics of chain initiation. Acetyl ACP (0−40 μM) was added to study the kinetics of chain elongation. Assays were initiated by addition of *holo*-ACP or malonyl CoA. Initial rates were measured from the slope of absorbance traces during the linear period. In act minimal PKS assays the linearity was maintained for 60 s; in the presence of MCAT the absorbance increased linearly for up to 5 min.

HPLC Activity Assays. HPLC quantification of SEK4 **2** and SEK4b **3** was performed in a Luna 5μ C_{18} (Phenomenex) column, 250 × 4.60 mm. The mobile phases for RP-HPLC: solvent A, water containing 0.05% trifluoroacetic acid (TFA); solvent B, acetonitrile containing 0.05% trifluoroacetic acid (TFA). The gradient consisted of the following steps: equilibration (0−5 min, 5%B); linear gradient (5−35 min, 5−75%B); linear gradient (35−36 min, 75−95%B); wash (36−39 min, 95%B); linear gradient (39−40 min, 95−5%B); and re-equilibration (40−45 min, 5%B). Purified SEK4 **2** and SEK4b **3** were used as standards (*7*, *29*). SEK4 **2** was detected after 19.1 min; SEK4b **3** was detected after 19.8 min.

RESULTS

DTT Degrades Acyl ACP Species. Acyl carrier proteins are translated as inactive *apo* proteins. *Holo*-ACP is generated by the attachment of phosphopantetheine (PP), derived from CoA, to a conserved serine residue. This reaction is catalyzed by *holo*-ACP synthase (ACPS) enzymes (*30*−*32*). Intermediate acyl groups are then attached to the terminal thiol of PP. Wild-type (WT) act *holo*-ACP can form an inactive intramolecular disulfide between the PP thiol and the thiol of cysteine-17 (*17*). This is prevented by use of a C17S mutant of act ACP (*17*). However, like other acyl carrier proteins, the C17S act *holo*-ACP can also form intermolecular disulfide dimers *via* the phosphopantetheinyl thiols, again rendering it inactive (*17*). Dithiothreitol (DTT) has been widely used to carry out the reduction of intermolecular disulfides, either prior to use of the ACP or *in situ*. In the case of act minimal PKS assays we have treated the ACP component with DTT and then removed the reducing agent by gel-filtration prior to assay (*20*, *21*). Because this is a time-consuming procedure, we (*7*, *17*) and others have used DTT *in situ* (*5*, *6*, *22*−*24*, *33*−*35*) at concentrations between 1 and 2 mM.

However, thiol reducing agents such as DTT are potentially incompatible with thiolesters because they could act as nucleophiles, decreasing intermediate thiolester concentrations by transthiolesterification and thus complicating kinetic analyses. Tris(2-carboxyethyl)phosphine (TCEP) has been used (*36*) as an alternative disulfide reductant with much attenuated nucleophilicity, especially for incubations of PKS components (*37*). We therefore investigated the reduction of *holo*-ACP dimers with DTT and TCEP by ESMS.

When act *holo*-ACP is lyophilized in air, it rapidly forms a disulfide dimer *via* the linking of the PP thiols. Dimer prepared in this way was then treated with DTT (1 mM) or TCEP (1 mM) in separate reactions at 30 °C (*7*). ESMS analysis indicated that over the course of 60 min, both reagents effectively reduce the disulfide and release monomeric *holo*-ACP (data not shown).

A comparison was then made of the effect of DTT and TCEP on the integrity of an acylated ACP by incubating malonyl-ACP (50 μM) with TCEP or DTT (1 mM) at 30 °C for 60 min. Incubation of malonyl-ACP (9527 Da) with DTT leads to transthiolesterification of the acyl group and production of *holo*-ACP (9441 Da) as observed by ESMS. We could only detect traces of malonyl-ACP after 60 min indicating almost complete removal of the malonate. This contrasts to the use of TCEP as reductant which does not react with malonyl ACP (Figure 2). Thus, stock solutions of *holo*-ACP were routinely treated with TCEP prior to all work described herein, and minimal PKS assays were performed in the presence of TCEP.

Kinetic Parameters for Malonyl CoA as a Substrate for Malonylation of act holo-ACP in Isolation. We have previously described a radioactive assay to study the kinetics of malonylation of *holo*-ACP with ^{14}C labeled malonyl CoA (*17*). The assay involves the reaction of ^{14}C-labeled malonyl CoA with *holo*-ACP over time. At set time points the reaction was sampled, protein precipitated, excess radiolabel washed away, protein resolubilized, and ^{14}C incorporation estimated by scintillation counting. In order to measure 7 protein concentrations, 7 malonyl CoA concentrations, in triplicate, required *ca*. 750 individual measurements and used significant amounts of pure protein. However, this enabled the kinetic parameters of $k_{cat} = 0.34$ min^{-1} and $K_M = 219$ μM to be measured (*17*). The assay was quantitative, but the very large number of measurements required and the use of large amounts of protein made this method impractical for further routine use.

More recently, continuous spectrophotometric methods to measure the release of CoA have been used by Molnos (*38*), Khandekar (*39*), and Liu (*40*) in reactions catalyzed by *Escherichia coli* MCAT, *Streptococcus pneumoniae* β-ke-

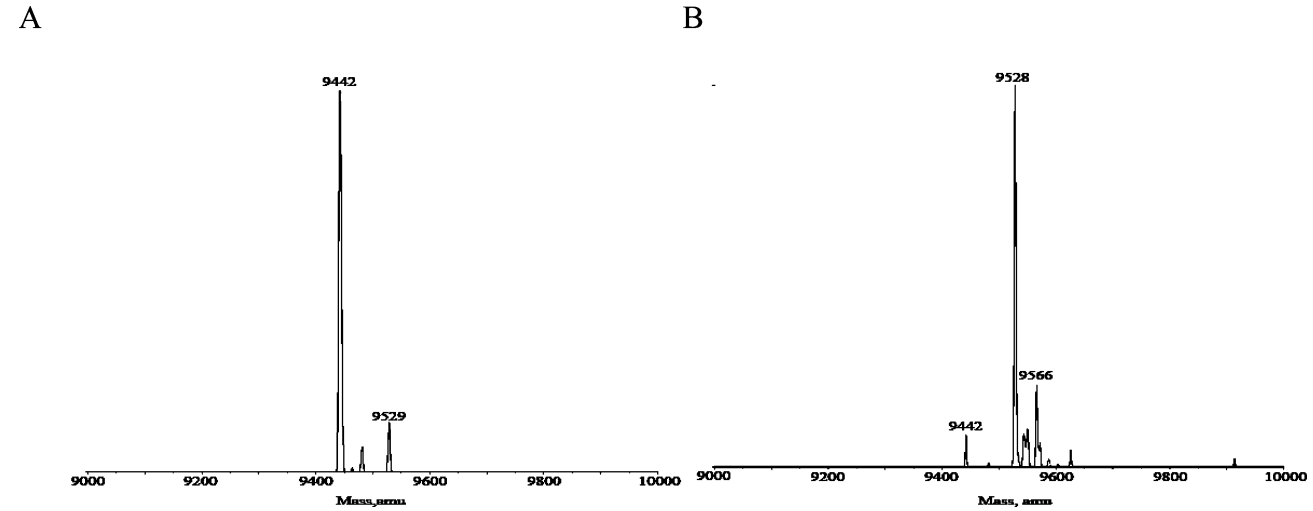
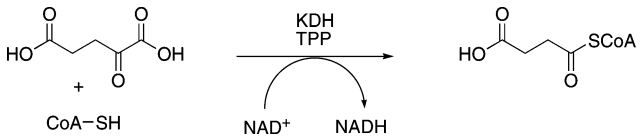


FIGURE 2: A: Incubation of malonyl ACP with DTT leads to production of *holo*-ACP (calculated mass, 9441 Da). Only traces of malonyl ACP (9527 Da) were observed. B: Incubation of malonyl ACP with TCEP showed the same ratio malonyl ACP:*holo*-ACP as the control experiments; i.e., >90% of the ACP species are malonylated (expected masses 9527, 9566 for malonyl ACP and the potassium adduct, respectively).

Scheme 2: Quantitative KDH Assay for CoASH



toacyl ACP synthase III (KASIII), and *Helicobacter pylori* MCAT, respectively. These assays couple the release of free CoASH to the oxidative decarboxylation of α-ketoglutaric acid (αKG), catalyzed by α-ketoglutarate dehydrogenase (KDH, Scheme 2). The free CoASH generated is used as a substrate by KDH to produce succinyl CoA with concomitant reduction of NAD^+ to NADH, which is followed spectrophotometrically at 340 nm ($\epsilon = 6230\ M^{-1}\ cm^{-1}$).

We applied this assay to the kinetics of the malonylation reaction of act *holo*-ACP. To ensure the coupling enzyme was in its *holo* form, KDH was incubated with αKG, TPP, $MgCl_2$, NAD^+, and malonyl CoA for 5 min before addition of the other components. Malonylation assays were then started by addition of purified act *holo*-ACP, and initial reaction rates (first 5−10% of reaction) were measured, for varied malonyl CoA and ACP concentrations, by observing the increase in NADH concentration at 340 nm (Figure 3A). Kinetic parameters were determined as $k_{cat} = 0.49 \pm 0.01\ min^{-1}$ and $K_M = 207 \pm 29\ \mu M$ for malonyl CoA (Figure 4A). An independent measure of k_{cat} ($0.45\ min^{-1}$) was made by varying ACP concentration at saturating malonyl CoA concentrations (Figure 4B).

Spectrophotometric Detection of act Minimal PKS Products. In order to measure the overall rate of the PKS, we directly measured the increase in concentration of the PKS products SEK4 **2** and SEK4b **3** by UV absorption. SEK4 **2** and SEK4b **3** were produced, purified, and quantified as previously described (*29*). The UV absorbance spectra of SEK4 and SEK4b show maxima in the aromatic region at 280 nm. However, CoA also absorbs strongly at this wavelength at pH = 7.3, and so 280 nm is unsuitable for determining changes in SEK concentration. On the other hand, a change in absorbance at 293 nm was only observed in the presence of malonyl CoA, *holo*-ACP, and $KS_\alpha KS_\beta$. Control reactions in which individual assay components were absent did not show any activity. The extinction coefficients of SEK4 **2** and SEK4b **3** at 293 nm were determined experimentally as 13 200 and 12 100 $M^{-1}\ cm^{-1}$ (100 mM phosphate buffer, pH = 7.3), respectively. We, and others, have shown by HPLC analysis of the products of actinorhodin minimal PKS assays that the ratio of compounds **2**:**3** is approximately 1:1 in act minimal PKS assays *in vitro* (*7*, *29*); thus, a mean extinction coefficient of 12 600 $M^{-1}\ cm^{-1}$ was used for quantifying kinetic measurements. The detection limit of polyketides by this method was determined to be 100 pmol by serial dilutions (corresponding to a change of 6 mAU at 293 nm under our experimental conditions).

Having established the detection conditions, we first investigated the dependence of the rate of octaketide production on the concentration of purified $KS_\alpha KS_\beta$ (Figure 3B, Figure 5). For $KS_\alpha KS_\beta$ concentrations of less than 0.5 μM, initial reaction rates increase linearly with the concentration of the KS components. In this concentration range, initial rates of octaketide production depend on the concentrations of *both* $KS_\alpha KS_\beta$ and *holo*-ACP. At higher $KS_\alpha KS_\beta$ concentrations (>0.5 μM), the reaction rate depends only on the concentration of *holo*-ACP, indicating that a *holo*-ACP dependent process is rate limiting. This agreed with our expectations that the synthesis of octaketides proceeds in at least two steps: i.e., malonylation of *holo*-ACP to form malonyl ACP, which is then used as a substrate by KS_α-KS_β.

Kinetic Parameters of Malonyl CoA as a Substrate for act holo-ACP in the Presence of $KS_\alpha KS_\beta$. Under conditions of high $KS_\alpha KS_\beta$ concentration (>3 μM), the self-malonylation of *holo*-ACP becomes rate limiting (Figure 5). Under these experimental conditions, the rate of production of octaketides must be exactly 1/8 of the malonylation rate. Thus, measurement of octaketide production can be used to measure the rate of malonylation of act *holo*-ACP in the presence of $KS_\alpha KS_\beta$. To do this, malonyl CoA concentrations were varied (at fixed ACP and $KS_\alpha KS_\beta$ concentrations) and the rate of octaketide production measured as described above. Kinetic parameters of $k_{cat} = 2.30 \pm 0.27\ min^{-1}$ and $K_M = 215 \pm 66\ \mu M$ for malonyl CoA were measured (Figure

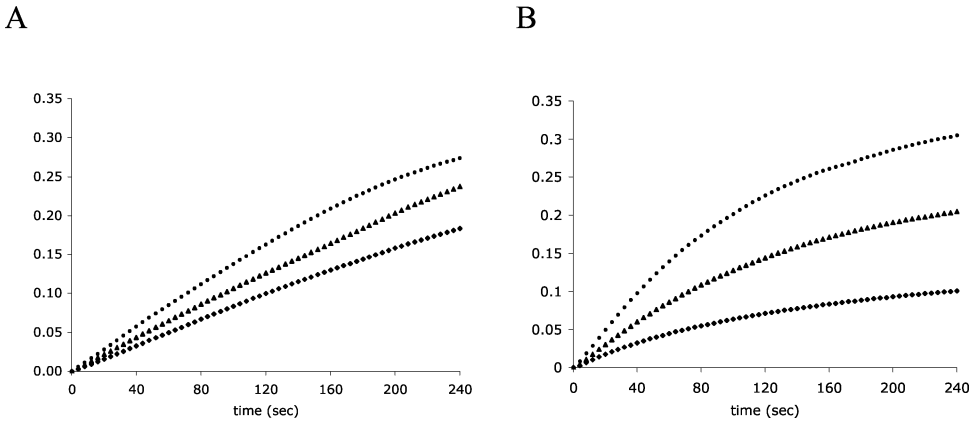


FIGURE 3: Raw kinetic data for KDH and $KS_{\alpha}KS_{\beta}$ assays. A. KDH assay, showing increase in NADH concentration (A_{340}) for self-malonylation of act *holo*-ACP at 15 (diamonds), 30 (triangles), and 50 μM (circles). B. $KS_{\alpha}KS_{\beta}$ assay showing increase in octaketide concentration (A_{293}) for assays containing act *holo*-ACP (80 μM), malonyl CoA (1 mM), and $KS_{\alpha}KS_{\beta}$ at 0.3 (diamonds), 0.5 (triangles), and 1.0 μM (circles).

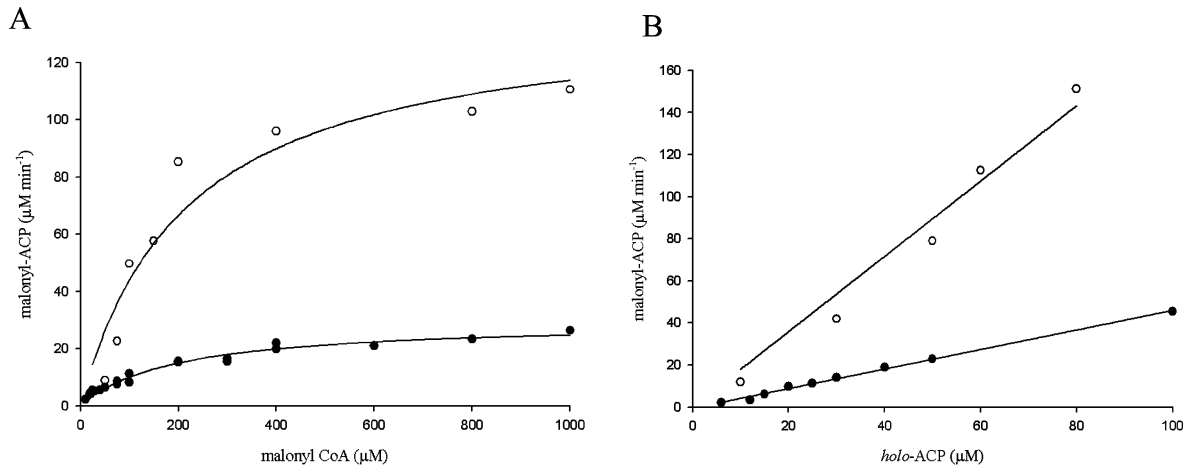


FIGURE 4: A: Determination of K_M and k_{cat} for the self-malonylation of *holo*-ACP using the KDH assay (filled circles, $K_M = 207$ μM) and the $KS_{\alpha}KS_{\beta}$ assay (open circles, $K_M = 216$ μM). *holo*-ACP concentration was fixed at 60 μM. B: Determination of k_{cat} for the self-malonylation of *holo*-ACP by the KDH assay (filled circles, $k_{cat} = 0.45$ min^{-1}) and the $KS_{\alpha}KS_{\beta}$ assay (open circles, $k_{cat} = 2.30$ min^{-1}). Malonyl CoA concentration was fixed at 1 mM (i.e., $> 5 \times K_M$).

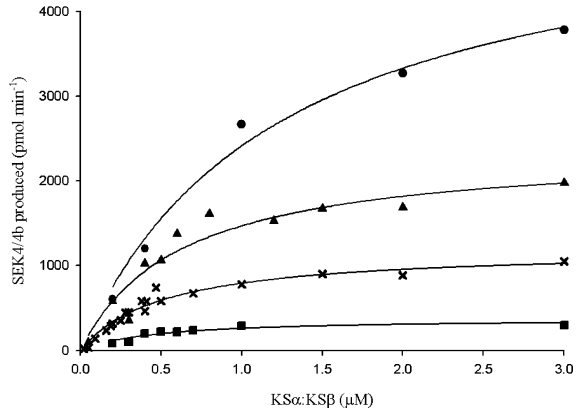


FIGURE 5: Dependence of initial rates of octaketide production on the concentration of $KS_{\alpha}KS_{\beta}$ at varying ACP concentrations: squares, 10 μM; crosses, 25 μM; triangles, 50 μM; circles, 80 μM.

4A). This experiment was repeated with a mutant of KS_{α}-KS_{β} in which the active site cysteine 169 of the KS_{α} component has been mutated to alanine (*20*). This mutant is incapable of polyketide biosynthesis, and thus the rate of malonylation could be measured using the KDH assay. In this assay k_{cat} was measured as 2.4 min^{-1}.

Kinetic Parameters for Malonyl ACP as a Substrate for $KS_{\alpha}KS_{\beta}$. The second step in the production of octaketides by the actinorhodin minimal PKS is the consumption of malonyl ACP by $KS_{\alpha}KS_{\beta}$. The rate of polyketide synthesis was studied by determining initial reaction rates of formation of octaketides at low $KS_{\alpha}KS_{\beta}$ concentrations and varying *holo*-ACP concentrations. A concentration of 0.3 μM KS_{α}-KS_{β} was chosen which lies within the linear range of the dependence of the rate with enzyme concentration (Figure 5), thus meaning that the polyketide synthesis step is rate limiting. Initial rates of reaction were then measured for a range of *holo*-ACP initial concentrations. A linear dependence of the rate on the concentration of *holo*-ACP is observed for ACP concentrations lower than 50 μM (Figure 6). This indicates that under these conditions an ACP-dependent process is the RLS, which agrees with the hypothesis that self-malonylation of ACP is the slowest step in the actinorhodin minimal PKS *in vitro*.

In order to measure the kinetic parameters for malonyl-ACP as a substrate for $KS_{\alpha}KS_{\beta}$, the rate of ACP malonylation was increased so that this was no longer the RLS. This was achieved by using a high concentration of *S. coelicolor* malonyl-CoA:ACP transacylase (MCAT), which catalyzes the rapid transfer of malonate from CoA onto ACP (*16*, *17*,

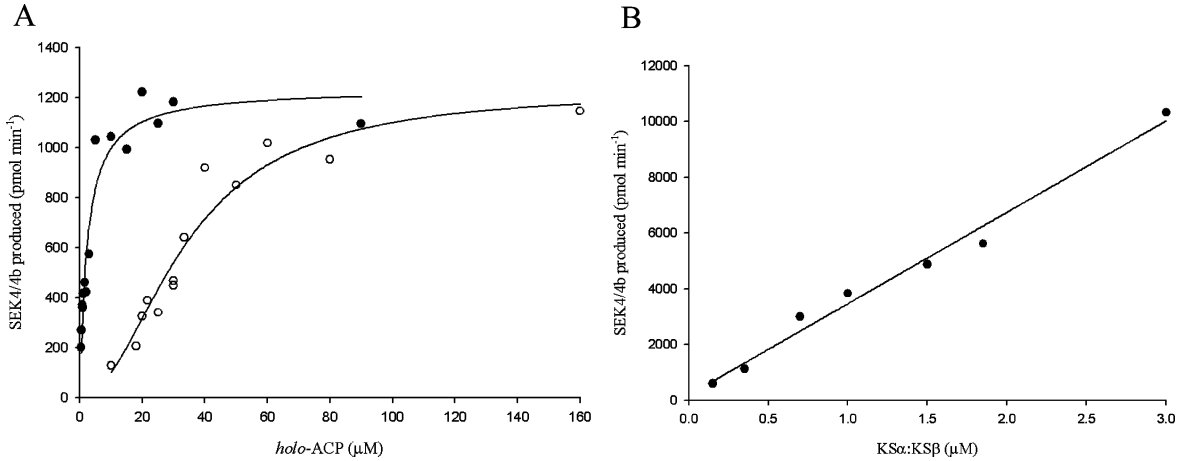


FIGURE 6: A: Dependence of the rate of octaketide production on *holo*-ACP concentration, with (filled circles) and without (open circles) the addition of MCAT. Michaelis−Menten kinetic parameters were estimated from the hyperbolic regression of the MCAT-catalyzed experiments. B: Dependence of initial reaction rates on the concentration KS_α/KS_β in MCAT-supplemented assays.

22). We therefore conducted assays at 0.3 μM $KS_\alpha KS_\beta$ with various concentrations of *holo*-ACP in the presence of MCAT (0.5 μM). Under these reaction conditions, the concentration of malonyl ACP in the assays is constant and equal to the initial, known concentration of *holo*-ACP. A comparison of the assays performed with and without MCAT is shown in Figure 6A. The same maximum velocity *V* is eventually achieved in both cases, as the catalysis by KS_α-KS_β is not affected by the malonylation of ACP. However, at low ACP concentrations the formation of octaketides is much faster in the presence of MCAT. This confirms our earlier observations using an HPLC assay (*7*). Kinetic parameters for the reactions catalyzed by $KS_\alpha KS_\beta$ with malonyl ACP were measured as $k_{cat} = 20.6 \pm 0.9$ min^{-1} and $K_M = 2.39 \pm 0.38$ μM.

To independently determine the turnover number (k_{cat}) of the ketosynthase, we measured the dependence of the reaction rate on the concentration of $KS_\alpha KS_\beta$ when MCAT (0.5 μM) was added to the assays. Initial rates are linear with increasing concentration of $KS_\alpha KS_\beta$ even at the highest concentration (3 μM) (Figure 6B). The catalytic constant k_{cat} was also independently calculated as 16.9 ± 0.9 min^{-1} from plots of rate of octaketide formation vs increasing $KS_\alpha KS_\beta$ concentration at saturating ACP and MCAT concentrations (Figure 6B).

Inhibition of the Actinorhodin Minimal PKS by act apo-ACP. *Apo*-ACP has previously been observed as an inhibitor of the act PKS (*22*) in the presence of MCAT. This inhibition could be due to inhibition of either the KS or MCAT components. We studied the inhibitory effect of act *apo*-ACP in PKS assays, constituted by MCAT, *holo*-ACP, and $KS_\alpha KS_\beta$. We first did an HPLC assay to detect the products of the act minimal PKS, following the procedure of Matharu (*7*). We thus incubated act *holo*-ACP with MCAT, malonyl CoA, and $KS_\alpha KS_\beta$, and *apo*-ACP was added to final concentrations of 100 and 200 μM. These assays indicated a reduction in the amount of octaketide production with increasing *apo*-ACP concentration. We then measured the rate of octaketide production by $KS_\alpha KS_\beta$ in the presence of varying concentrations of act *apo*-ACP (0−200 μM) and act *holo*-ACP (2.5−25 μM). These assays show that act *apo*-ACP is a mixed inhibitor with $K_{ic} = 50$ μM $\pm$ 12 μM and $K_{iu} = 137$ μM $\pm$ 28 μM (Figure 7). Assays were then performed to determine whether the rate decrease was due to inhibition of MCAT. Thus the rate of ACP malonylation was measured using the KDH assay in the presence of MCAT as described above. No inhibition of MCAT by act *apo*-ACP was observed.

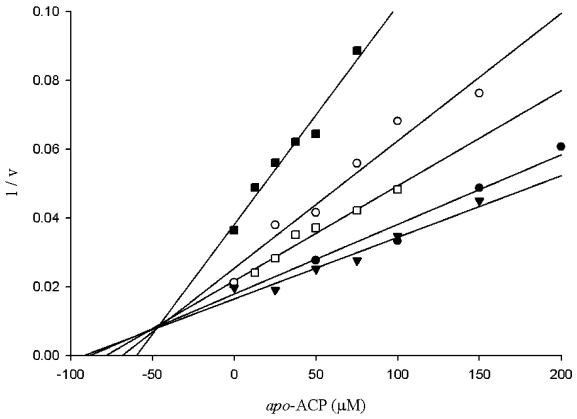


FIGURE 7: Inhibition of act PKS by act *apo*-ACP. Plot of 1/v vs [I]. *Holo*-ACP concentrations are as follows: full squares, 2.5 μM; open circles, 5 μM; open squares, 7.5 μM; full circles, 15 μM; triangles, 25 μM.

Initiation Assay. In the presence of a sufficient concentration of MCAT, malonylation of ACP is no longer the RLS of the act minimal PKS. The measured rate under these conditions now corresponds to a new RLS which could be any of the succeeding reactions: decarboxylation of malonyl ACP to form acetyl ACP; chain extension; chain release. In order to determine whether decarboxylation to form starter unit (initiation) could be the RLS under these conditions, we measured the rate of octaketide production in the presence of increasing concentrations of acetyl ACP (Figure 8). Actinorhodin C17S acetyl ACP was produced by incubating *holo*-ACP with acetyl imidazole and monitoring the reaction by ESMS (*25*). When full acetylation had been achieved, the acetyl ACP was purified by gel-filtration chromatography.

Under conditions where the PKS was rate limiting (i.e., in the presence of MCAT), rate enhancements of the minimal PKS were observed when acetyl ACP was added. At the highest concentration of acetyl ACP, the measured k_{cat}, using malonyl ACP as a substrate, increases 3-fold (Figure 8).

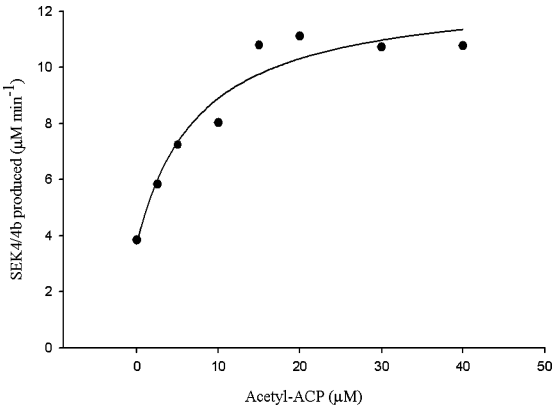


FIGURE 8: Effect of addition of acetyl ACP on the rate of octaketide production by the minimal act PKS + MCAT.

DISCUSSION

The actinorhodin minimal PKS, consisting of *holo*-ACP, KS_α, and KS_β, catalyzes at least 18 separate chemical reactions *in vitro* (Scheme 1). These reactions consist of a loading step where malonate is transferred from CoA to the terminal thiol of the ACP phosphopantetheine. This is followed by a chain initiation step consisting of decarboxylation of malonyl ACP to form acetyl ACP. Seven more loading and extension cycles must be followed by cyclization and release to form the octaketides SEK4 **2** and SEK4b **3**. While the rates of malonyl transfer have been measured for the initial loading reaction in the presence and absence of MCAT (*16*, *17*), and overall rates of polyketide formation have been measured (*7*, *22*−*24*), no investigations have been described which measure the rates of any of the intervening reactions, or indeed, have determined which steps are rate limiting under differing conditions.

We initially developed a sensitive continuous quantitative spectrometric assay for the production of the octaketides **2** and **3**. This allows the collection of genuine initial rate data under conditions where less than 10% of the starting materials are consumed. Using this assay, and varying both ACP and $KS_\alpha KS_\beta$ concentrations it is clear that malonylation of ACP is the RLS for the act minimal PKS *in vitro* (Figure 5). We also showed that the reductant TCEP is more suitable in these assays than DTT which degrades malonyl ACP species (Figure 2). This in accord with recent studies of other PKS enzymes *in vitro* (*37*).

We have developed three assays for measuring the self-malonylation of act ACP. In previous work we described the use of a discontinuous assay using ^{14}C-labeled malonate as a substrate to measure the kinetic parameters for this reaction (*17*). Here, however, two new assays have been developed. In order to improve the sensitivity and reproducibility of the measurements, we modified the KDH assay described by Molnos and others (*38*−*40*) to quantify the rate of released CoASH when *holo*-ACP reacts with malonyl CoA (Scheme 1). Once again, this assay allows the measurement of initial rates under conditions where less than 10% of the malonyl CoA and *holo*-ACP is consumed and from which kinetic parameters can be easily determined ($k_{cat} = 0.49 \pm 0.01$ min^{-1} and $K_M = 207 \pm 29$ μM). The KDH assay gives similar results to the ^{14}C-malonyl CoA assay ($k_{cat} = 0.34$ min^{-1}, $K_M = 219$ μM) (*17*), although the absolute rate of malonylation is 44% faster than previously measured. This is probably due to our previous use of DTT in the ^{14}C-malonyl CoA assay which would have reduced the concentration of malonyl ACP.

The third assay for malonylation of ACP relies on measuring the rate of octaketide production by the complete minimal act PKS under conditions when malonylation of ACP is the RLS. Under these conditions, octaketides are produced at 1/8 the rate of malonyl-transfer, so measuring the rate of octaketide production by the PKS complex gives the rate of malonyl transfer in the complex. In these assays the rate of malonyl transfer is consistently higher than the rate of malonyl transfer when $KS_\alpha KS_\beta$ is not present (k_{cat} for self-malonylation 0.49 min^{-1}, k_{cat} for malonylation in the presence of $KS_\alpha KS_\beta$ 2.30 min^{-1}). However, the K_M value for malonyl CoA remains constant (*ca* 210 μM) for both processes. The elevated k_{cat} value was independently confirmed by using the KDH assay to directly measure the rate of malonylation of act *holo*-ACP in the presence of a mutant of $KS_\alpha KS_\beta$ incapable of chain extension. Under these circumstances, k_{cat} for malonylation of ACP was 2.4 min^{-1}, a 5-fold increase over the malonylation of ACP alone.

It is conceivable that the increase in observed rate of malonylation of ACP in the presence of $KS_\alpha KS_\beta$ could be accounted for if $KS_\alpha KS_\beta$ were contaminated with *S. coelicolor* MCAT, although this has not been reported in previous studies (*22*). If $KS_\alpha KS_\beta$ were contaminated with MCAT, then, under conditions where malonylation is rate limiting, one would expect to observe a decrease in the measured K_M value toward that for the MCAT-catalyzed reaction (K_M of malonyl CoA as a substrate for MCAT is estimated *ca.* 30 μM) (*34*). However, the same K_M value for malonyl CoA is measured in the presence and in the absence of $KS_\alpha KS_\beta$. Thus, we conclude that the increase in measured k_{cat} value represents a genuine increase in the catalytic efficiency of the ACP-malonylation reaction when the ACP is in the presence of $KS_\alpha KS_\beta$.

The exact mechanism for self-malonylation of Type II PKS acyl carrier proteins has not yet been determined. It is therefore interesting to observe a 5-fold increase in malonylation rate in the presence of $KS_\alpha KS_\beta$. It is tempting to speculate that when bound to $KS_\alpha KS_\beta$ the ACP can take up a conformation which optimizes self-malonylation, although structural studies do not suggest that act ACP takes up multiple distinct conformations in solution (*41*). Alternatively, specific residues on the surface of $KS_\alpha KS_\beta$ may assist in malonyl transfer. It has been previously speculated that the KS_α component of the PKS could contain an acyl transferase GHS catalytic motif (*2*), although site-directed mutagenesis studies do not support this idea (*42*, *43*) and crystallographic data show that these residues are not surface exposed (*44*). In either event, the kinetic evidence presented here suggests that *holo*-ACP is closely associated with $KS_\alpha KS_\beta$ during the malonylation step. Similar results were gathered by us previously using an HPLC assay which suggested that $KS_\alpha KS_\beta$ sequesters ACP from solution (*7*).

Regardless of the fact that the rate of malonyl transfer increases when $KS_\alpha KS_\beta$ is present, the malonylation of the act minimal PKS is the RLS *in vitro*. This is in agreement with our previous observations using an HPLC-based assay (*7*) but may also explain why expression of the tetracenomycin (tcm) Type II ACP on high copy-number vectors in *Streptomyces glaucescens* results in increased production of

tcm-D3 *in vivo* (*45*) and why increases in act ACP concentrations *in vivo* also increase the yield of polyketides in *S. coelicolor* (*46*).

However, if a high concentration of *S. coelicolor* MCAT is added to the minimal PKS, loading accelerates and is no longer rate limiting. Malonyl ACP is now the substrate for the ensuing reactions, and varying the ACP concentration allows the measurement of kinetic parameters for malonyl-ACP as a substrate for the $KS_\alpha KS_\beta$-catalyzed reactions. Under these conditions, it appears that chain initiation (i.e., decarboxylation of malonyl ACP) becomes rate limiting because the addition of exogenous acetyl ACP increases the rate still further (Figure 8). Under these conditions the measured parameters for malonyl CoA as a substrate for $KS_\alpha KS_\beta$-catalyzed reactions are $k_{cat} = 20.6 \pm 0.9$ min^{-1} and $K_M = 2.39 \pm 0.38$ μM. Thus k_{cat} for chain initiation is over 10-fold higher than malonylation of ACP in the presence of $KS_\alpha KS_\beta$. Titration of acetyl CoA into this system suggests an approximate K_M for acetyl CoA of 5 μM.

Previous attempts have been made to measure kinetic parameters for Type II PKS complexes. For example, Khosla and co-workers have studied both homologous and heterologous PKS complexes consisting of the act $KS_\alpha KS_\beta$ paired with WT act ACP and frenolicin ACP (*22*), as well as $KS_\alpha KS_\beta$ proteins from the Tcm, Sch, and Pms clusters paired with the Fren, Zhu, Gra, WhiE, and Pms acyl carrier proteins (*23*). In all cases, these assays have relied upon the addition of MCAT and are thus comparable with the assays described herein. However, the previously reported k_{cat} values ranged between 0.11 min^{-1} and 0.95 min^{-1}, compared with 20.6 min^{-1} measured here. In all cases the previously described work used DTT as the reductant, and it is likely that malonyl-ACP concentrations were below the total ACP concentration in these cases. This is partially explained by our observation of a 44% increase in the rate constant for malonylation of ACP when TCEP is used as the reductant vs DTT (0.49 min^{-1} vs 0.34 min^{-1}). A further difference is that our continuous assays indicate linearity of data over only the first 120 s of reaction under typical reaction conditions (Figure 3A) and previously reported assays, including our own (*7*), over longer time periods may have underestimated initial rates.

We also examined the effect of *apo*-ACP on the minimal PKS (Figure 8). Previous workers have reported that *apo*-ACP inhibits the minimal PKS in the presence of MCAT (*22*). We measured precise kinetic parameters for inhibition and showed that inhibition is due to binding of *apo*-ACP to $KS_\alpha KS_\beta$ rather than to MCAT. *Apo*-ACP shows a mixed pattern of inhibition, suggesting that it can bind at more than one ACP binding-site. As $KS_\alpha KS_\beta$ binds malonyl ACP for two different reactions (initiation and extension), it seems likely that the observed pattern of inhibition represents *apo*-ACP binding at the extension (i.e., KS_α) and initiation (i.e., KS_β) sites. K_{ic} for inhibition by *apo*-ACP is 50 μM, a factor of 20-fold higher than K_M for malonyl ACP as a substrate (2.39 μM). This difference indicates that $KS_\alpha KS_\beta$ recognizes malonyl ACP preferentially, indicating either a specific binding interaction to malonyl-phosphopantetheine, or that malonyl ACP takes up a different conformation to *apo*-ACP. The latter idea may be valid because malonyl CoA and CoA itself do not appear to inhibit this step.

We then investigated whether under fast malonylation conditions, and in the presence of excess acetyl ACP, chain extension or chain termination steps (i.e., cyclization and release) were now rate limiting. In an attempt to observe PKS intermediates bound to ACP prior to cyclization and release, we conducted assays at high $KS_\alpha KS_\beta$ concentrations. This was required because the number of polyketide chains under construction at any given time point is unlikely to significantly exceed the number of active synthases; thus, by increasing the synthase concentration we hoped to observe part-completed acylated ACPs by ESMS. However, increasing $KS_\alpha KS_\beta$ concentrations also increases the overall rate of octaketide production and malonyl CoA consumption, so aliquots of reaction mixtures containing $KS_\alpha KS_\beta$, *holo*-ACP, MCAT, and malonyl CoA were rapidly quenched after initiation of reaction and examined by ESMS and HPLC. Only **2**, **3**, and malonyl ACP species were observed, indicating no accumulation of part-complete polyketide chains attached to ACP, suggesting that cyclization and release steps are faster than extension steps.

Consideration of the individual rate constants measured for the individual catalytic steps shows an increase in value for each succeeding step. Thus self-malonylation of ACP in the absence of $KS_\alpha KS_\beta$ is relatively slow ($k_{cat} = 0.49$ min^{-1}). This increases 5-fold when $KS_\alpha KS_\beta$ is present ($k_{cat} = 2.30$ min^{-1}). Malonyl ACP is consumed by $KS_\alpha KS_\beta$ to produce octaketides with a yet-higher rate constant ($k_{cat} = 20.6$ min^{-1}) which most likely represents the rate of initiation. In the presence of an excess of acetyl ACP, the rate increases 3-fold more ($k_{cat} \approx 60$ min^{-1}), probably representing the rate of chain extension. We were unable to measure rates for the cyclization and release steps, but these are likely higher still. Thus, the act minimal PKS appears to be optimized for the conversion of malonyl CoA to octaketides without the accumulation of intermediate acyl ACP species. It also appears that under circumstances where malonyl ACP is available at concentrations above its K_M as a substrate for $KS_\alpha KS_\beta$ (2.4 μM), the rate of chain initiation, i.e., decarboxylation of malonyl-ACP to provide the required acetyl ACP starter unit, is rate limiting. This makes metabolic sense because it ensures that acetyl ACP is not wasted.

A key question raised by our *in vitro* results is their relevance to the situation *in vivo*. It is clear that the minimal act PKS consisting of ACP, KS_α, and KS_β is competent to produce octaketides over a wide range of protein and substrate (malonyl CoA) concentrations (Figures 4−6), but does it do this efficiently? In the absence of MCAT the RLS is the malonylation of ACP. The rate of this reaction depends on both the concentrations of malonyl CoA and *holo*-ACP and increases *linearly* with *holo*-ACP concentration (Figure 4B). Figure 6 shows how the overall rate of octaketide production depends on *holo*-ACP concentration. At concentrations above about 80 μM *holo*-ACP the rate of malonylation is sufficient to match or outpace the requirement for malonyl-ACP by $KS_\alpha KS_\beta$. MCAT, of course, speeds up malonyl transfer, and for concentrations of *holo*-ACP below about 80 μM, the presence of MCAT speeds up the overall reaction.

Figure 9 shows how the behavior of the MCAT-catalyzed and the self-catalyzed malonylation reactions vary with the concentration of ACP, MCAT, and malonyl CoA. The kinetic parameters for act *holo*-ACP as a substrate for MCAT cannot

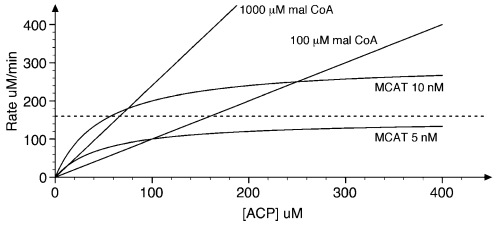


FIGURE 9: Comparison of the behavior of MCAT catalyzed malonylation of *holo*-ACP vs self-acylation of act PKS *holo*-ACP. Linear plots show how the rate of self-malonylation of *holo*-ACP in the presence of $KS_\alpha KS_\beta$ varies with ACP concentration at two concentrations of malonyl CoA (rate data from Figure 4). Curves show the effect of saturation catalysis by MCAT at differing MCAT concentrations (for simulated MCAT with K_M of 50 μM for ACP and k_{cat} of 500 s^{-1} at saturating malonyl CoA). Dashed line shows demand for malonyl ACP by $KS_\alpha KS_\beta$ at 1 μM.

be measured because of the self-malonylation reaction, but by comparison with other ACPs (*16*) it is reasonable to estimate a K_M of 50 μM and k_{cat} of 500 s^{-1}. It is clear that under some conditions, high MCAT concentration (>10 nM), low ACP concentration (<80 μM), and low malonyl CoA concentration (<100 μM), the MCAT catalyzed reaction would be expected to dominate. However it is also possible for the self-malonylation reaction to dominate, for example at high ACP (>80 μM) and malonyl CoA concentrations (>400 μM) and low MCAT concentrations (<5 nM).

The *in vivo* concentrations of malonyl CoA and act *holo*-ACP have never been measured in *S. coelicolor*, but Rock and co-workers have estimated the concentration of *E. coli* FAS ACP as between 17 and 130 μM *in vivo*, and malonyl CoA concentrations have been estimated between 110 and 1000 μM (*47*). Few *in vivo* experiments have been performed to correlate individual protein concentrations with the rate of polyketide biosynthesis. However, Khosla, Hopwood, and co-workers showed in an elegant series of experiments that likely increases of *in vivo* ACP concentrations correlate well with the yield of polyketides (*46*). This is in agreement with similar observations of Hutchinson and co-workers using the tetracenomycin PKS (*45*). The fact that ACP appears to be limiting *in vivo* correlates well with observations reported here and elsewhere (*22*) that ACP concentration is limiting *in vitro* up to around 100 μM (Figure 6A).

MCAT concentrations are also unknown *in vivo*. Studies of the *S. coelicolor* MCAT *in vitro* have used concentrations between 0.5 nM (*16*) and 100 nM (*22*). If *in vivo* MCAT concentrations were at the lower end of this range, then our results indicate that self-malonylation would be dominant and malonylation of the ACP would be the RLS *in vivo*. If, however, MCAT concentrations were at the upper end of the range, then MCAT-catalyzed malonylation would dominate, and provision of acetyl ACP for use as a starter unit would be rate limiting for the PKS.

The demand for malonyl ACP must also be considered as well as the supply. Our results show that in the absence of excess acetyl ACP, octaketides are produced by $KS_\alpha KS_\beta$ at 20.6 min^{-1} which corresponds to the use of malonyl ACP at 165 min^{-1}. For a $KS_\alpha KS_\beta$ concentration of 1 μM (typical for *in vitro* studies), this corresponds to a malonylation rate of 165 $\mu M \cdot min^{-1}$; this is shown as the dashed line in Figure 9. It is clear that this could be supplied by ACP at approximately 80 μM and malonyl CoA at 1000 μM in the absence of MCAT or by 60 μM ACP and saturating malonyl CoA in the presence of 10 nM MCAT. Thus, the concentrations of all catalytic and substrate participants are required in order to determine which pathway dominates *in vivo*. While the results reported here show which factors are important and give limits for each process, further experiments will be required to determine these concentrations *in vivo* during actinorhodin biosynthesis.

In conclusion, the results presented here show that individual catalytic steps of the act PKS can be dissected. Rate constants measured for each succeeding step increase in magnitude, ensuring that acetyl ACP is not accumulated as an intermediate and that acyl ACP species are processed quickly to products. Furthermore, the assays reveal that the act PKS selectively binds acylated ACPs over *apo*-ACP despite the fact that there is little structural difference between these species. The dissection of the individual steps will allow direct comparison with other iterative PKS systems such as those involved in lovastatin (*12*) and norsolorinic acid biosynthesis (*13*) where efforts to engineer 'Type II' systems from Type I native proteins have been recently reported. In current experiments, the continuous assays are being used to probe the ACP-selectivity of the minimal PKS, investigate the intriguing 5-fold increase in efficiency of self-malonylation in the presence of $KS_\alpha KS_\beta$, and dissect the effect of specific mutations on individual catalytic steps.

ACKNOWLEDGMENT

The authors thank the School of Chemistry and University of Bristol.

REFERENCES

1. Malpartida, F., and Hopwood, D. A. (1984) Molecular-cloning of the whole biosynthetic-pathway of a *Streptomyces* antibiotic and its expression in a heterologous host, *Nature 309*, 462−464.
2. Fernandez-Moreno, M. A., Martinez, E., Boto, L., Hopwood, D. A., and Malpartida, F. (1992) Nucleotide-sequence and deduced functions of a set of cotranscribed genes of *Streptomyces-coelicolor A3(2)* including the polyketide synthase for the antibiotic actinorhodin, *J. Biol. Chem. 267*, 19278−19290.
3. McDaniel, R., Ebert-Khosla, S., Fu, H., Hopwood, D. A., and Khosla, C. (1994) Engineered biosynthesis of novel polyketides - influence of a downstream enzyme on the catalytic specificity of a minimal aromatic polyketide synthase, *Proc. Natl Acad. Sci. U.S.A. 91*, 11542−11546.
4. McDaniel, R., Ebert-Khosla, S., Hopwood, D. A., and Khosla, C. (1993) Engineered biosynthesis of novel polyketides, *Science 262* (5139), 1546−1550.
5. Carreras, C. W., Pieper, R., and Khosla, C. (1996) Efficient synthesis of aromatic polyketides *in vitro* by the actinorhodin polyketide synthase, *J. Am. Chem. Soc. 118*, 5158−5159.
6. Carreras, C. W., and Khosla, C. (1998) Purification and in vitro reconstitution of the essential protein components of an aromatic polyketide synthase, *Biochemistry 37*, 2084−2088.
7. Matharu, A. L., Cox, R. J., Crosby, J., Byrom, K. J., and Simpson, T. J. (1998) MCAT is not required for *in vitro* polyketide synthesis in a minimal actinorhodin polyketide synthase from *Streptomyces coelicolor*, *Chem. Biol. 5*, 699−711.
8. Hertweck, C., Luzhetskyy, A., Rebets, Y., and Bechthold, A. (2007) Type II polyketide synthases: gaining a deeper insight into enzymatic teamwork, *Nat. Prod. Rep. 24*, 162−190.
9. Grimm, A., Madduri, K., Ali, A., and Hutchinson, C. R. (1994) Characterization of the *Streptomyces peucetius* ATTC-29050 genes encoding doxorubicin polyketide synthase, *Gene, 151*, 1−10.
10. Cox, R. J., Glod, F., Hurley, D., Lazarus, C. M., Nicholson, T. P., Rudd, B. A. M., Simpson, T. J., Wilkinson, B., and Zhang, Y. (2004) Rapid cloning and expression of a fungal polyketide synthase gene involved in squalestatin biosynthesis, *Chem. Commun.* 2260−2261.

11. Cox, R. J. (2007) Polyketides, proteins and genes in fungi: programmed nano-machines begin to reveal their secrets, *Org. Biomol. Chem. 5*, 2010−2026.
12. Ma, S. M., and Tang, Y. (2007) Biochemical characterization of the minimal polyketide synthase domains in the lovastatin nonaketide synthase LovB, *FEBS J. 274*, 2854−2864.
13. Crawford, J. M., Dancy, B. C. R., Hill, E. A., Udwary, D. W., and Townsend, C. A. (2006) Identification of a starter unit acyl-carrier protein transacylase domain in an iterative type I polyketide synthase, *Proc. Natl. Acad. Sci. U.S.A. 103*, 16728−16733.
14. Khosla, C., and Harbury, P. B. (2001) Modular enzymes, *Nature 409*, 247−252.
15. Revill, W. P., Bibb, M. J., and Hopwood, D. A. (1995) Purification of a malonyltransferase from *Streptomyces coelicolor* and analysis of its genetic determinant, *J. Bacteriol. 177*, 3946−3952.
16. Szafranska, A. E., Hitchman, T. S., Cox, R. J., Crosby, J., and Simpson, T. J. (2002) Kinetic and mechanistic analysis of the malonyl CoA:ACP transacylase from *Streptomyces coelicolor* indicates a single catalytically competent serine nucleophile at the active site, *Biochemistry 41*, 1421−1427.
17. Hitchman, T. S., Crosby, J., Byrom, K. J., Cox, R. J., and Simpson, T. J. (1998) Catalytic self-acylation of Type II polyketide synthase acyl carrier proteins, *Chem. Biol. 5*, 35−47.
18. Arthur, C., Cox, R. J., Crosby, J., Rahman, M. M., Simpson, T. J., Soulas, F., Spogli, R., Szafranska, A. E., Westcott, J., and Winfield, C. J. (2002) Synthesis and characterisation of acyl carrier protein bound polyketide analogues, *ChemBioChem 3*, 253−257.
19. Arthur, C. J., Szafranska, A., Evans, S. E., Findlow, S. C., Burston, S. G., Owen, P., Clark-Lewis, I., Simpson, T. J., Crosby, J., and Crump, M. P. (2005) Self-malonylation is an intrinsic property of a chemically synthesized Type II polyketide synthase acyl carrier protein, *Biochemistry 44*, 15414−15421.
20. Bisang, C., Long, P. F., Cortes, J., Westcott, J., Crosby, J., Matharu, A. L., Cox, R. J., Simpson, T. J., Staunton, J., and Leadlay, P. F. (1999) A chain initiation factor common to both modular and aromatic polyketide synthases, *Nature 401*, 502−505.
21. Nicholson T. P., Winfield, C., Wescott, J., Crosby, J., Simpson, T. J., and Cox, R. J. (2003) First *in vitro* directed biosynthesis of new compounds by a minimal Type II polyketide synthase: evidence for the mechanism of chain length determination, *Chem. Commun.* 686−687.
22. Dreier, J., Shah, A. N., and Khosla, C. (1999) Kinetic analysis of the actinorhodin aromatic polyketide synthase, *J. Biol. Chem. 274*, 25108−25112.
23. Lee, T. S., Khosla, C., and Tang, Y. (2005) Orthogonal protein interactions in spore pigment producing and antibiotic producing polyketide synthases, *J. Antibiot. 58*, 663−666.
24. Tang, Y., Lee, T. S., Kobayashi, S., and Khosla. C. (2003) Ketosynthases in the initiation and elongation modules of aromatic polyketide synthases have orthogonal acyl carrier protein specificity, *Biochemistry 42*, 6588−6595.
25. Crosby, J., Byrom, K., Hitchman, T. S., Cox, R. J., Crump, M. P., Findlow, S. C., Bibb, M. J., and Simpson, T. S. (1998) Acylation of *Streptomyces* Type II polyketide synthase acyl carrier proteins, *FEBS Lett. 433*, 132−138.
26. Arthur, C. J., Szafranska, A. E., Long, J., Mills, J., Cox, R. J., Findlow, S. C., Simpson, T. J., Crump, M. P., and Crosby, J. (2006) The malonyl transferase activity of Type II Polyketide Synthase Acyl Carrier Proteins, *Chem. Biol. 13*, 587−596.
27. Crosby, J., Sherman, D. H., Bibb, M. J., Revill, W. P., Hopwood, D. A., and Simpson, T. J. (1995) Polyketide synthase acyl carrier proteins from *Streptomyces* − expression in *Escherichia coli*, purification and partial purification, *Biochim. Biophys. Acta 1251*, 32−42.
28. Winston, R. L., and Fitzgerald, M. C. (1998) Concentration and desalting of protein samples for mass spectrometry analysis, *Anal. Biochem. 262*, 83.
29. Fu, H., Hopwood, D. A., and Khosla, C. (1994) Engineered biosynthesis of novel polyketides: evidence for temporal, but not Regiospecific, control of cyclization of an aromatic polyketide precursor, *Chem. Biol. 1*, 205−210.
30. Lambalot, R. H., Gehring, A. M., Flugel, R. S., Zuber, P., LaCelle, M., Marahiel, M. A., Reid, R., Khosla, C., and Walsh, C. T. (1996) A new enzyme superfamily - the phosphopantetheinyl transferases, *Chem. Biol. 3*, 923−936.
31. Cox, R. J., Hitchman, T. S., Byrom, K. J., Findlow, S. C., Tanner, J. A., Crosby, J., and Simpson, T. J. (1997) Post-translational modification of heterologously expressed *Streptomyces* Type II polyketide synthase acyl carrier proteins, *FEBS Lett. 405*, 267−272.
32. Cox, R. J., Crosby, J., Daltrop, O., Glod. F., Jarzabek, M. E., Nicholson, T. P., Reed, M., Simpson, T. J., Smith, L. H., Soulas, F., Szafranska, A. E., and Westcott, J. (2002) *Streptomyces coelicolor* phosphopantetheinyl transferase: a promiscuous activator of polyketide and fatty acid synthase acyl carrier proteins, *J. Chem. Soc., Perkin Trans. 1*, 1644−1649.
33. Shen, B., and Hutchinson, C. R. (1993) Enzymatic synthesis of a bacterial polyketide from acetyl and malonyl coenzyme A, *Science 262*, 1535−1540.
34. Zhou, P., Florova, G., and Reynolds, K. A. (1999) Polyketide synthase acyl carrier protein (ACP) as a substrate and a catalyst for malonyl ACP biosynthesis, *Chem. Biol. 6*, 577−584.
35. Bao, W., Wendt-Pienkowski, E., and Hutchinson, C. R. (1998) Reconstruction of the iterative Type II polyketide synthase for tetracenomycin F2 biosynthesis, *Biochemistry 37*, 8132−8138.
36. Burmeister Getz, E., Xiao, M., Chakrabarty, T., Cooke, R., and Selvin, P. (1999) A comparison between the sulphydryl reductants tris(2-carboxyethyl)phosphine and dithiothreitol for use in protein biochemistry, *Anal. Biochem. 273*, 73−80.
37. Izumikawa, M., Cheng, Q., and Moore, B. S. (2006) Priming Type II polyketide synthase via a Type II nonribosomal peptide synthetase mechanism, *J. Am. Chem. Soc. 128*, 1428−1429.
38. Molnos, J., Gardiner, R., Dale, G. E., and Lange, R. (2003) A continuous coupled enzyme assay for bacterial malonyl-CoA:acyl carrier protein transacylase (FabD), *Anal. Biochem. 319*, 171−176.
39. Khandekar, S. S., Gentry, D. R., Van Aller, G. S., Warren, P., Xiang, H., Silverman, C., Doyle, M. L., Chambers, P. A., Konstantinidis, A. K., Brandt, M., Daines, R. A., and Lonsdale, J. T. (2001) Identification, substrate specificity, and inhibition of the *Streptococcus pneumoniae β-ketoacyl-acyl carrier protein synthase III (FabH), J. Biol. Chem. 272*, 30024−30030.
40. Liu, W., Han, C., Hu, L., Chen, K., Shen, X., and Jiang, H. (2006) Characterization and inhibitor discovery of one novel malonyl CoA:acyl carrier protein transacylase (MCAT) from *Helicobacter pylori*, *FEBS Lett. 580*, 697−702.
41. Crump, M. P., Crosby, J., Dempsey, C. E., Parkinson, J. A., Murray, M., Hopwood, D. A., and Simpson, T. J. (1997) Solution structure of the actinorhodin polyketide synthase acyl carrier protein from *Streptomyces coelicolor* A3(2), *Biochemistry 36*, 6000−6008.
42. Kim, E. S., Cramer, K. D., Shreve, A. L., and Sherman, D. H. (1995) Heterologous expression of an engineered biosynthetic-pathway - functional dissection of Type-II polyketide synthase components in *Streptomyces* Species, *J. Bacteriol. 177*, 1202−1207.
43. Meurer, G., and Hutchinson, C. R. (1995) Functional Analysis of putative *β*eta-ketoacyl-acyl carrier protein synthase and acyltransferase active-site motifs in a Type-II polyketide synthase of *Streptomyces glaucescens*, *J. Bacteriol. 177*, 477−481.
44. Keating-Clay, A. T., Maltby, D. A., Medzihradszky, K. F., Khosla, C., and Stroud, R. M. (2004) An antibiotic factory caught in action, *Nature Struct. Mol. Biol. 11*, 888−893.
45. Decker, H., Summers, R. G., and Hutchinson, C. R. (1994) Overproduction of the acyl carrier protein component of a Type II polyketide synthase stimulates production of tetracenomycin biosynthetic intermediates in Streptomyces glaucescens, *J. Antibiot. 47*, 54−63.
46. Khosla, C., McDaniel, R., Ebert-Khosla, S., Torres, R., Sherman, D. H., Bibb, M. J., and Hopwood, D. A (1993) Genetic construction and functional analysis of hybrid polyketide synthases containing heterologous acyl carrier proteins, *J. Bacteriol. 175*, 2197−2204.
47. Heath, R. J., and Rock, C. O. (1996) Inhibition of *β*-ketoacyl-acyl carrier protein synthase III (FabH) by acyl-acyl carrier protein in *Escherichia coli*, *J. Biol. Chem. 271*, 10996−11000.

BI701784C