MCAT is not required for *in vitro* polyketide synthesis in a minimal actinorhodin polyketide synthase from *Streptomyces coelicolor*

Anne-Lise Matharu, Russell J Cox, John Crosby, Kate J Byrom and Thomas J Simpson

Background: It has been proposed that *Streptomyces* malonyl CoA:*holo* acyl carrier protein transacylases (MCATs) provide a link between fatty acid and polyketide biosynthesis. Two recent studies have provided evidence that the presence of MCAT is essential for polyketide synthesis to proceed in reconstituted minimal polyketide synthases (PKSs). In contrast to this, we previously showed that the *holo* acyl carrier proteins (ACPs) from type II PKSs are capable of catalytic self-malonylation in the presence of malonyl CoA, which suggests that MCAT might not be necessary for polyketide biosynthesis.

Results: We reconstituted a homologous actinorhodin (act) type II minimal PKS *in vitro*. When act *holo*-ACP is present in limiting concentrations, MCAT is required by the synthase complex in order for polyketide biosynthesis to proceed. When *holo*-ACP is present in excess, however, efficient polyketide synthesis proceeds without MCAT. The rate of polyketide production increases with *holo*-ACP concentration, but at low ACP concentration or equimolar ACP:KS:CLF (KS, ketosynthase; CLF, chain length determining factor) concentrations this rate is significantly lower than expected, indicating that free *holo*-ACP is sequestered by the KS/CLF complex.

Conclusions: The rate of polyketide biosynthesis is dictated by the ratio of *holo*-ACP to KS and CLF, as well as by the total protein concentration. There is no absolute requirement for MCAT in polyketide biosynthesis *in vitro*, although the role of MCAT during polyketide synthesis *in vivo* remains an open question. MCAT might be responsible for the rate enhancement of malonyl transfer at very low free *holo*-ACP concentrations or it could be required to catalyse the transfer of malonyl groups from malonyl CoA to sequestered *holo*-ACP.

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

Correspondence: Thomas J Simpson
E-mail: tom.simpson@bris.ac.uk

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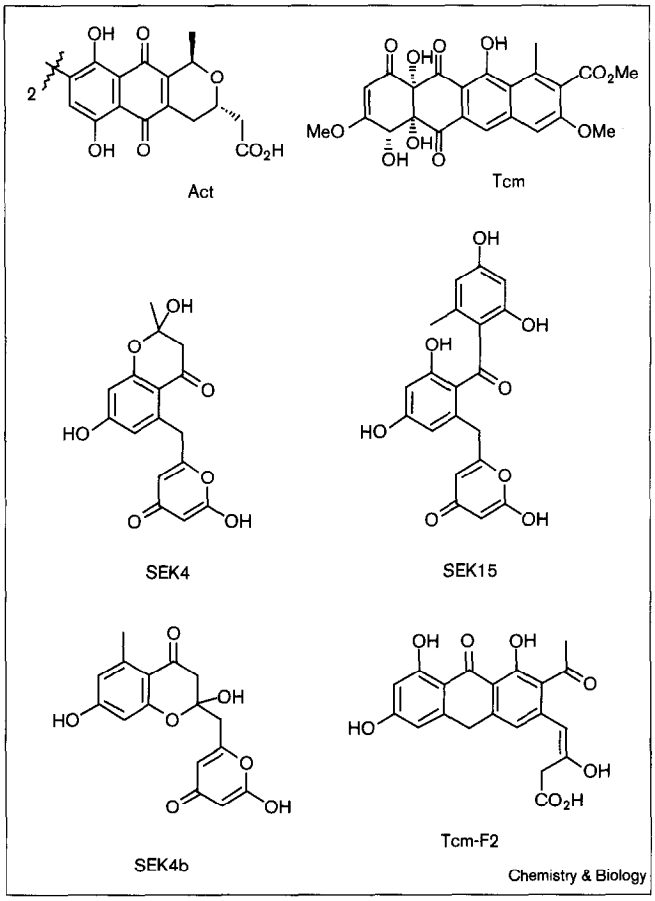
Introduction

The enzymes that govern the assembly of polyketides by microorganisms are receiving increasing attention as access to them has improved through molecular genetic methods [1]. In particular, the biosynthesis of polycyclic aromatic compounds such as actinorhodin (act) and tetracenomycin (tcm) (Figure 1) by *Streptomyces* type II polyketide synthases (PKSs) has been studied extensively *in vivo* [2–4] and, more recently, *in vitro* [5–7]. Experiments by Hutchinson, Khosla and others have shown that expression of 'minimal PKS' systems consisting of open reading frames (ORFs) coding for β-keto acyl synthase (KS), chain length factor (CLF) and acyl carrier proteins (ACP) produce protein complexes that are capable of synthesising polyketides such as the octaketides SEK4 and SEK4b (act genes) and the decaketide SEK15 (tcm genes) *in vivo*. These experiments have been carried out in *Streptomyces* species in which endogenous PKS genes are missing or have been deleted. The *in vivo* experiments are unable to determine whether a functioning minimal PKS expressed in a streptomycete is interacting with any other endogenous proteins, however. Of particular interest in this respect are proteins involved in the parallel pathway of fatty acid biosynthesis. [*Act*I ORF2 is a protein of significant similarity to *act*I ORF1, which encodes the β-keto acyl ACP synthase (KS). *Act*I ORF2, however, does not possess an active-site cysteine essential for KS activity. Although essential for PKS activity, the precise role of the *act*I ORF2 gene product is unknown. Early *in vivo* 'mix and match' expression experiments suggested that this protein controlled final polyketide chain length, hence the name 'chain length factor (CLF)'. More recent experiments have shown that chain length is controlled in a more complex way by interactions between the *act*I ORF1 and *act*I ORF2 gene products as well as other proteins [8]. The alternative nomenclature systems of KS_α (KS) and KS_β (CLF) is no less unsatisfactory as the actI ORF2 gene product is clearly not a KS. 'CLF' will remain in use here until a better understanding of this protein's role is forthcoming.]

Figure 1



Structures of polyketides produced by type II polyketide synthases.

Fatty acids are biosynthesised in gram-negative and gram-positive bacteria using classical type II fatty acid synthase (FAS) systems. In *Escherichia coli*, malonyl ACP is the component that provides malonyl extender groups for growing fatty acids, which are themselves held as acyl thiolesters of ACP [9]. Malonyl ACP formation from malonyl CoA is catalysed by malonyl CoA : *holo*-ACP transacylase (MCAT) [10–12]. A similar set of FAS genes has been shown to exist in *Streptomyces coelicolor*. The putative *S. coelicolor fab* cluster contains genes homologous to the *E. coli* FAS ketoacylsynthases (KAS) I and III, ACP and MCAT, and the cluster would seem to consist of all the genes necessary for fatty acid biosynthesis. In contrast, the *act* cluster of *S. coelicolor*, which contains all the genes required for actinorhodin biosynthesis, does not seem to code for activities that might be thought necessary for synthesis. Although KS and ACP genes are present, there is no ORF that codes for an MCAT homologue. In the case of the *act* cluster, early sequencing results suggested a putative general acyl transferase (AT) domain (GHSXG) as part of *act*I ORF1; other homologous ORFs, such as *tcm*K (KS), contain similar motifs [13]. Mutagenesis experiments using both *act*I ORF1 [14] and *tcm*K [15] showed the putative active-site serine to be unnecessary for polyketide synthesis. In the case of daunorubicin (dps), a discrete gene has been identified (*dps*D) that is homologous to an acyl transferase. Even though this gene is part of the dps PKS cluster, it is not required for polyketide biosynthesis. Experiments involving deletion of *dps*D showed that the correct starter propionyl unit was still incorporated into the polyketide [16,17]. Thus, although AT-like domains have been found in a small number of type II PKS clusters, their function *in vivo* is uncertain. This is a surprising result, in view of the large number of AT domains required for polyketide biosynthesis in type I modular systems such as erythromycin and rapamycin [18], as well as the absolute requirement for MCAT during fatty acid biosynthesis.

Isolation and characterisation of *Streptomyces* MCAT

MCAT was isolated by Revill and Hopwood [19] from extracts of *S. coelicolor* using an assay based on the transfer of ^{14}C-malonyl groups from ^{14}C-malonyl CoA to wild-type act *holo*-ACP. Purification of MCAT led to protein sequencing and eventual location of *fabD* (MCAT) on the *S. coelicolor* chromosome. Sequencing indicated high homology to the *E. coli* FAS MCAT originally isolated by Williamson and Wakil [10] using *E. coli* FAS ACP. Contemporaneously Hutchinson and coworkers [20,21] reported similar work using *Streptomyces glaucescens* from which MCAT was isolated and purified, using the tcm ACP expressed and purified separately from *S. glaucescens*. The conclusions of Revill and Hutchinson and coworkers [19–21] led to the proposal that MCAT could also catalyse the malonyl transfer required for efficient polyketide biosynthesis *in vivo*.

In order to examine further the biochemistry of *S. coelicolor* MCAT we have cloned and expressed it in *E. coli* as a His_6 fusion protein (T.S Hitchman, R.J.C., J.C. and T.J.S, unpublished observations). The overexpressed protein was purified to homogeneity and characterised in detail. As expected, the purified protein catalyses the transfer of malonyl groups from malonyl CoA to *S. coelicolor* FAS ACP. The reaction between *S. coelicolor* MCAT and *E. coli* ACP is also catalysed, although the specificity (k_{cat}/K_M) of MCAT for the *E. coli* ACP is lower.

Self-malonylation of *holo*-ACP

In light of the above results we were surprised, during the course of the above studies with overexpressed His_6-MCAT, to observe that rigorously purified act *holo*-ACP can rapidly and catalytically self-malonylate in the presence of malonyl CoA [22]. We have extensively characterised this reaction and have found that the MCAT has no measurable effect except at limiting *holo*-ACP concentrations. In contrast to the type II PKS ACPs, the FAS ACPs examined did not show this property. Our results of the study of PKS ACP self-acylation showed a typical kinetic profile with a reaction rate that increases with malonyl CoA

concentration, with eventual saturation at high malonyl CoA concentrations. The measured K_M of about 200 μM for malonyl CoA in the self-malonylation reaction, and k_{cat} value of 0.34 min^{-1} suggested that this self-catalysed malonylation could account for polyketide synthesis without the need for MCAT activity and hence account for the lack of this activity in type II PKS gene clusters. Additionally, we investigated the substrate specificity of self-acylation and show that the transfer of various β- and γ-oxo acyl groups (e.g. methylmalonyl, acetoacetyl and succinyl) is possible, whereas self-catalytic transfer of straight chain saturated acyl groups (e.g. acetyl and butyryl) is not.

Reconstruction of minimal PKS systems *in vitro*

More recent work has focused on the reconstitution of active type II PKS systems *in vitro*. Carreras and Khosla [23] used a heterologous system containing act KS/CLF and frenolicin (fren) ACP. Although crude preparations were capable of polyketide biosynthesis, this ability was lost on further purification of KS/CLF, enabling purification of a protein subsequently shown to be MCAT from *S. coelicolor* (based on its ability to restore PKS activity to the *in vitro* system). Khosla and Walsh and coworkers [5] have also reported the reconstruction of heterologous PKS systems using act KS/CLF obtained as a semi-pure fraction from *S. coelicolor* CH999 pSEK38. Fren and granaticin (gra). *Apo*-ACPs were treated with *holo*-ACP synthetase (ACPS) and acyl CoAs to form >95% acylated *holo*-ACPs. Fren acetyl ACP prepared in this way was shown to be an effective starter unit for polyketide synthesis.

Hutchinson and coworkers [24] have also recently reported the *in vitro* reconstitution of a type II minimal PKS. Their strategy differed from that of Carreras and Khosla [23] in that KS/CLF was expressed separately from ACP. Using the tcm minimal genes *tcmK* (KS) and *tcmL* (CLF) with *tcmM* (ACP), together with the cyclase *tcmN*, a reconstituted assay produced the polyketide tcmF2 (Figure 1) when supplemented with malonyl CoA and MCAT. Bao *et al.* [24] did observe a level of self-catalysed malonyl transfer to *tcmM* ACP, although the rate was significantly lower than that catalysed by MCAT. These results are in agreement with our previously reported results showing self-acylation of act *holo*-ACP in the presence of malonyl CoA. Despite the initial claim by these authors that MCAT is an 'absolute requirement' for polyketide production, it is clear that self-malonylation of *holo*-ACP could account for a proportion (up to 10% of the reported levels) of the polyketide produced.

The absence of MCAT genes in the type II PKS clusters, and the evidence that MCAT stimulates polyketide production *in vitro*, has led to the proposal that endogenous *Streptomyces* FAS MCAT proteins perform the role of loading malonyl groups onto the PKS ACPs. Our recent results suggested that self-malonylation could account for polyketide biosynthesis by minimal PKS systems and therefore that MCAT might not be required for the activity of type II minimal PKS proteins [22]. Here, we examine further the requirement for *S. coelicolor* MCAT in a reconstituted homologous act minimal PKS. In doing so, we address a number of points of apparent contradiction between our results and those of others. The results re-open the question of whether MCAT is essential for polyketide biosynthesis *in vivo* and begin to probe the exact roles of ACP and MCAT.

Results

Expression and purification of *holo*-ACPs

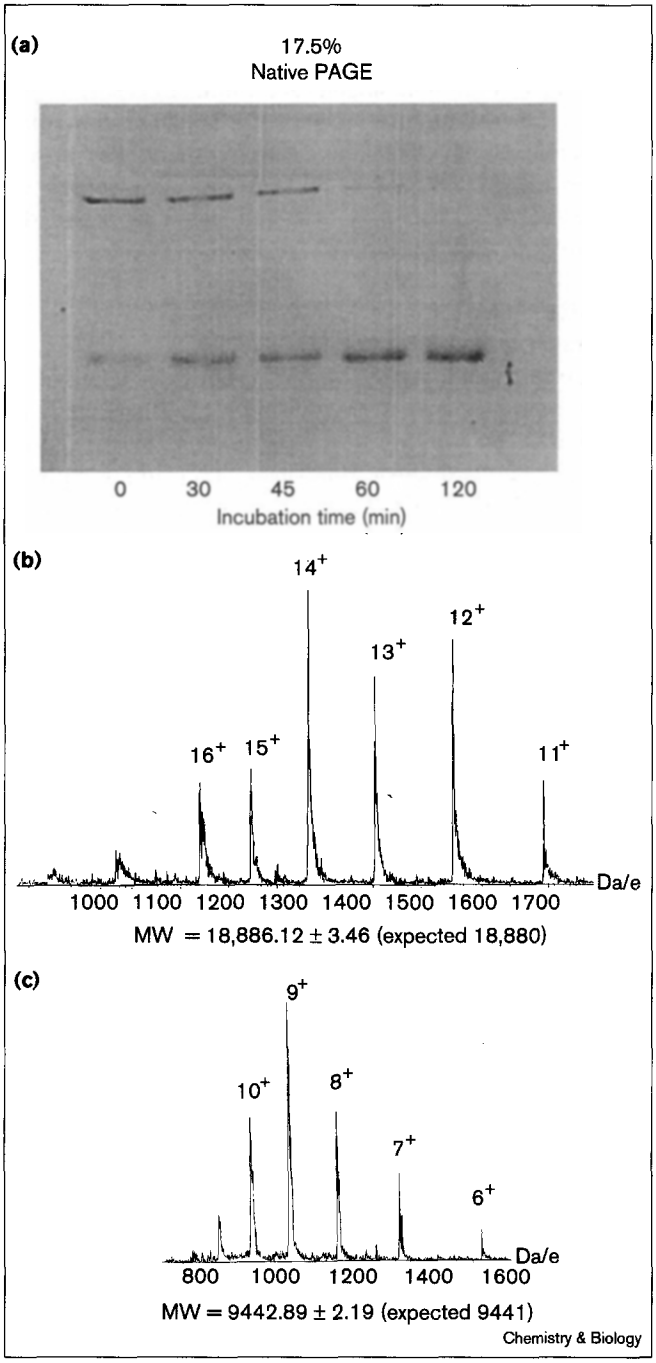
Acyl carrier proteins were purified from their appropriate *E. coli* expression strains according to published methods [25]. The ACPs were shown to be free from *E. coli* MCAT using procedures discussed previously [22]. Importantly, it was ensured that ACPs used for the work described below were present in >95% active *holo* form containing the 4′-phosphopantetheine prosthetic group attached as a phosphoester to the essential serine (Ser42) residue. This was achieved through the co-expression of ACP with *E. coli holo*-ACP synthetase (ACPS), as previously discussed [26], which is necessary to obtain high levels of active *holo* protein. Inactive *apo* protein was removed using chromatography.

It was necessary also to ensure that the *holo*-ACPs were present in the correct form. We previously demonstrated rapid disulphide formation between the thiols of the 4′-phosphopantetheine of *holo*-ACPs to form dimers in buffers containing insufficient (less than 1mM) dithiothreitol (DTT) [26]. In the case of wild-type act ACP, rapid formation of an internal disulphide between the 4′-phosphopantetheine and Cys17 also occurs: we surmounted this particular problem by the formation of a Cys17→Ser (C17S) mutant [27]. We found that sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS PAGE) cannot separate ACP monomers and disulphide dimers, so we utilised native PAGE, as well as electrospray mass spectroscopy (ESMS), to demonstrate that the ACPs were present as the active *holo* monomers (Figure 2 for act C17S *holo*-ACP data). As controls, disulphide dimers of the *holo*-ACPs were formed either by freeze drying, or by desalting against a buffer without DTT using a sepharose column. When freeze-dried *holo*-ACPs are resuspended in buffers containing 1mM DTT, monomerisation occurs, but on a slow time scale such that even after 45 min significant amounts of dimer remain. Increasing the amount of DTT can speed up the process (data not shown).

Reconstitution of polyketide biosynthesis *in vitro*

After initial cloning work in *E. coli*, a clone was constructed in pIJ4122 that consisted of an initial His_6 sequence followed by *act*I ORF1 (KS) and *act*I ORF2 (CLF). The putative translational coupling between ORFs 1 and 2 was not altered [13]. After transformation

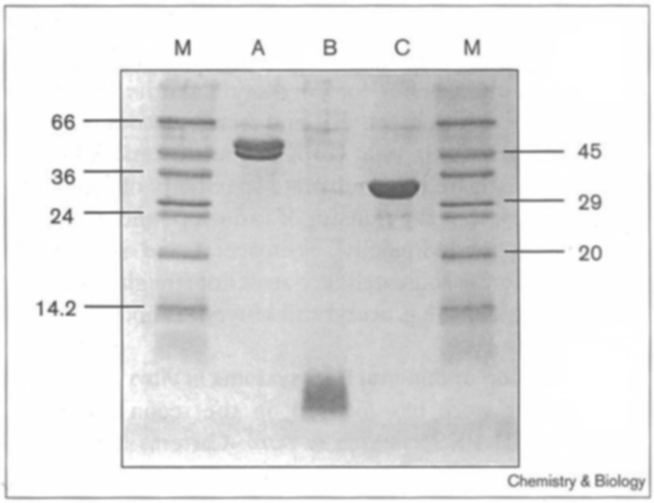
Figure 2



Slow monomerization of act C17S *holo*-ACP in 1mM DTT at 30°C. **(a)** 17.5% native PAGE showing act C17S *holo*-ACP dimer (top band) being converted to a monomeric species over 120 min. **(b)** ESMS of pure act C17S *holo*-ACP dimer showing the envelope of charged species. **(c)** ESMS of pure act C17S *holo*-ACP monomer showing the envelope of charged species.

of this vector (pRJC006) into *S. coelicolor* CH999 [28] (a strain lacking the *act* cluster entirely) protein production was induced by supplementation with thiostrepton to 5 μg/ml. As expected, large amounts of the target protein were produced (> 30 mg/l). Rapid cell lysis and purification

Figure 3



Purified proteins used in this study on 15% SDS PAGE. Lanes: M, marker (from top) 66 kDa, 45 kDa, 36 kDa, 29 kDa, 24 kDa, 20 kDa, 14.2 kDa; A, KS/CLF; B, wild-type act *holo*-ACP monomer; C, His_6-MCAT.

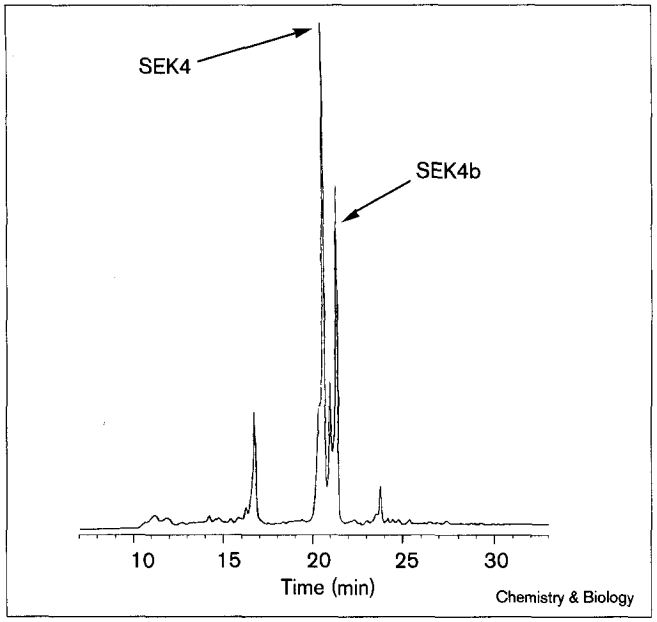
using Ni^{2+} affinity chromatography resulted in a protein extract containing the act KS and CLF in apparent homogeneity (Figure 3).

We had *apo* and *holo* forms of both wild-type and C17S act ACP at our disposal, as well as commercially available *E. coli* ACP. Additionally, by controlling the purification procedures the *holo*-ACPs were available as monomers or intramolecular disulphides or dimeric intermolecular disulphides. Purified ACPs were added at known concentrations to purified KS and CLF, with or without malonyl CoA as described below. Assays were incubated for the indicated times at 30°C and worked up by quenching with potassium phosphate and then extraction into ethyl acetate. The solvent was then removed and the residue examined using reverse-phase high-performance liquid chromatography (RP-HPLC) (Figure 4). In order to calibrate the HPLC integrals SEK4 and SEK4b were purified from *S. coelicolor* CH999pSEK4 and obtained as homogenous solids before injection of known masses onto the HPLC column.

The KS/CLF protein preparation does not contain *S. coelicolor* MCAT or other transacylase activity

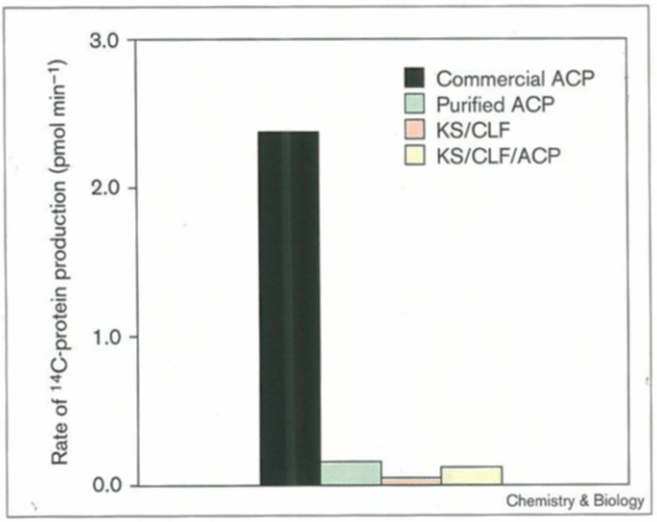
We have previously shown that *E. coli* FAS ACP is a good substrate for *S. coelicolor* MCAT and can be used as a potential assay for detecting MCAT activity in protein fractions (T.S Hitchman, R.J.C., J.C and T.J.S, unpublished observations). Commercially available *E. coli* ACP (>60% purity) appears to contain substantial MCAT activity, as incubation with ^{14}C-labelled malonyl CoA alone resulted in formation of labelled protein. This activity was removed

Figure 4



A typical HPLC trace showing compounds detected at 280 nm after incubation of act *holo*-ACP monomer with malonyl CoA and KS/CLF under the conditions described in the Materials and methods section.

Figure 5



Rate of formation of ^{14}C-labelled protein in the presence of ^{14}C-malonyl CoA (22 μM, 1.7 GBq mmol^{-1}). Experiments were carried out in the presence of 50 μM *E. coli* ACP and 2.5 μM act KS/CLF.

by purifying the ACP by our usual procedures. Ion-exchange chromatography, followed by desalting yielded protein with substantially lower acyltransferase activity (Figure 5). Incubation of this purified ACP with ^{14}C-malonyl CoA and the KS/CLF fraction resulted in formation of labelled protein at a very low rate. Incubation of KS/CLF alone with ^{14}C-malonyl CoA resulted in formation of labelled protein at an even lower rate.

Holo ACP monomer is required for polyketide biosynthesis

All current evidence points to the 4′-phosphopantetheine thiol of *holo*-ACP as the point of attachment for both the malonyl group and the growing polyketide chain. *Holo*-ACP disulphide dimer should not, therefore, be able to participate in polyketide biosynthesis. Polyketides were produced when *holo*-ACP monomer was added in the presence of malonyl CoA (Figure 4). The dimer was formed by resuspending *holo*-ACP in buffer not containing DTT. When added to the KS/CLF in the presence of 1mM malonyl CoA and 1mM DTT polyketides were formed, but at a rate significantly below that observed for the *holo*-ACP monomer (Figure 6). Presumably monomer, liberated from the dimer by slow reaction with DTT, was able to participate in the reaction. No polyketides (SEK4 and SEK4b) were produced when *apo*-ACP or *E. coli* FAS *holo*-ACP was used in the same assay.

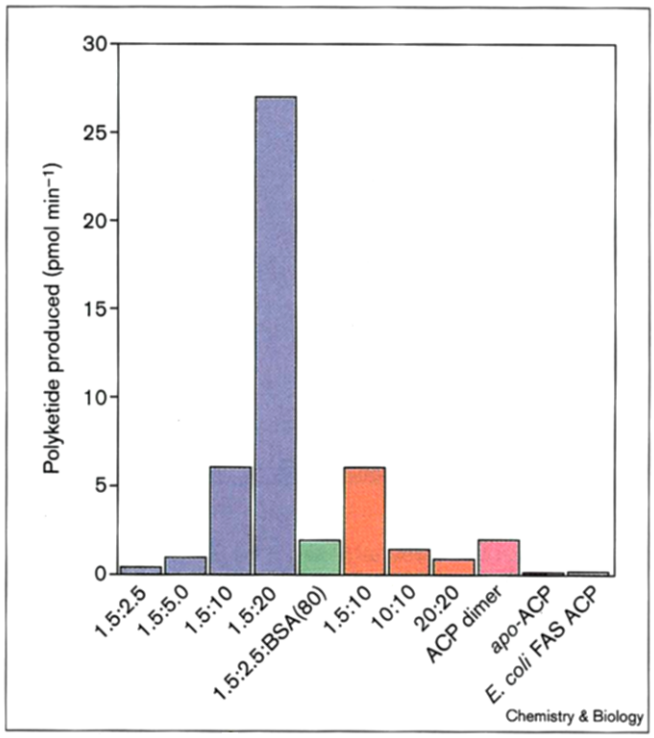
Time course of polyketide production

In order to determine the best time frame for studying polyketide production, we examined the variation of polyketide production with time. Parallel 100 μl reactions were set up that contained KS/CLF (1.5 μM), wild-type act *holo*-ACP monomer (50 μM), MCAT (1 μM) and malonyl CoA (1 mM). Reactions were initiated by the addition of malonyl CoA and stopped at time points over 120 min. SEK4 and SEK4b production was assayed using calibrated HPLC and plotted versus time (Figure 7). After 120 min, a total of 4.0 nmol (40 μM in 100 μl reaction) of polyketide had been produced. Because SEK4 and SEK4b are octaketides we expected 12.5 nmol of polyketide products (based on one eighth of the 100 nmol malonyl CoA available). Although only 4.0 nmol was produced, the figure shows that polyketide production was still continuing at a significant rate at 120 min. The production curve remains essentially linear up to around 20 min and subsequent assays containing up to 50 μM ACP were performed on this time scale, with faster assays containing >50 μM ACP being performed for shorter times.

Requirement for MCAT during polyketide biosynthesis

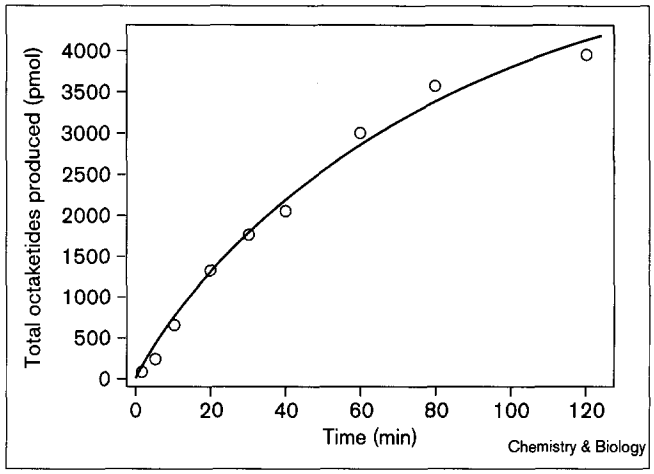
As expected, incubation of purified KS/CLF (1.5 μM) with malonyl CoA (1 mM), with or without the addition of MCAT (1 μM), did not result in detectable polyketide (SEK4 or SEK4b) production. We estimate the limits of our assay to be < 5 pmol of SEK4 or SEK4b, indicating any production of these compounds to be occurring at less than 0.25 pmol min^{-1} in standard 20 min assays. Addition of purified wild-type act *holo*-ACP monomer (2.5 μM) in the presence of MCAT (1 μM) restored polyketide production. When the experiment was repeated in the absence of MCAT very low, but detectable, polyketide production was observed. Addition of further wild-type act *holo*-ACP monomer led to increased levels

Figure 6



Rate of polyketide (SEK4 + SEK4b) production with varying protein concentrations (first figure in ratio refers to KS/CLF concentration, μM; the second figure to ACP concentration, μM). Blue bars, rate of polyketide production with increasing wild-type *holo*-act ACP monomer concentration at 1.5 μM KS/CLF. Green bar, enhancement in rate with general protein concentration increase. Red bars, effect of increasing KS/CLF concentrations. Magenta bars, use of other ACP species as indicated (at 50 μM).

Figure 7



Time course of a reaction containing 1 mM malonyl CoA, 50 μM wild-type act *holo*-ACP monomer, 1 μM His_6-MCAT and 1.5 μM KS/CLF. The maximum expected yield of octaketides is 12.5 nmol; 4.0 nmol was produced after 2 h.

of SEK4 and SEK4b, however. At low ACP concentration, that is, in the same region as the KS/CLF (<10 μM), the rate of polyketide production appeared to be significantly less than that observed in the presence of MCAT. Above a *holo*-ACP monomer concentration of 20 μM no difference in the rate of polyketide production was observed (Figure 8a), however.

Effects of varying protein concentration on polyketide production

Examination of the effect of varying wild-type act *holo*-ACP monomer concentration was performed by addition of *holo*-ACP up to 200 μM in the absence of MCAT (Figure 8b). The rate of polyketide production increased with increasing *holo*-ACP monomer concentration. There appeared to be no indication of saturation even at the highest total *holo*-ACP concentration used (200 μM).

In order to examine this effect more fully we incubated equimolar amounts of act *holo*-ACP and KS/CLF at higher concentrations (Figure 6). Reactions containing 10 μM of each of ACP/KS/CLF produced polyketides at a significantly lower rate than reactions containing 1.5 μM of KS/CLF and 10 μM ACP. Similarly reactions containing 20 μM concentrations of all protein components produced polyketides at a similarly reduced rate. The effect of total protein concentration was also examined. Addition of bovine serum albumin (BSA) to 80 μM to a standard reaction containing 1.5 μM KS/CLF and 2.5 μM act *holo*-ACP led to a slightly increased rate of polyketide production of compared with reactions lacking BSA.

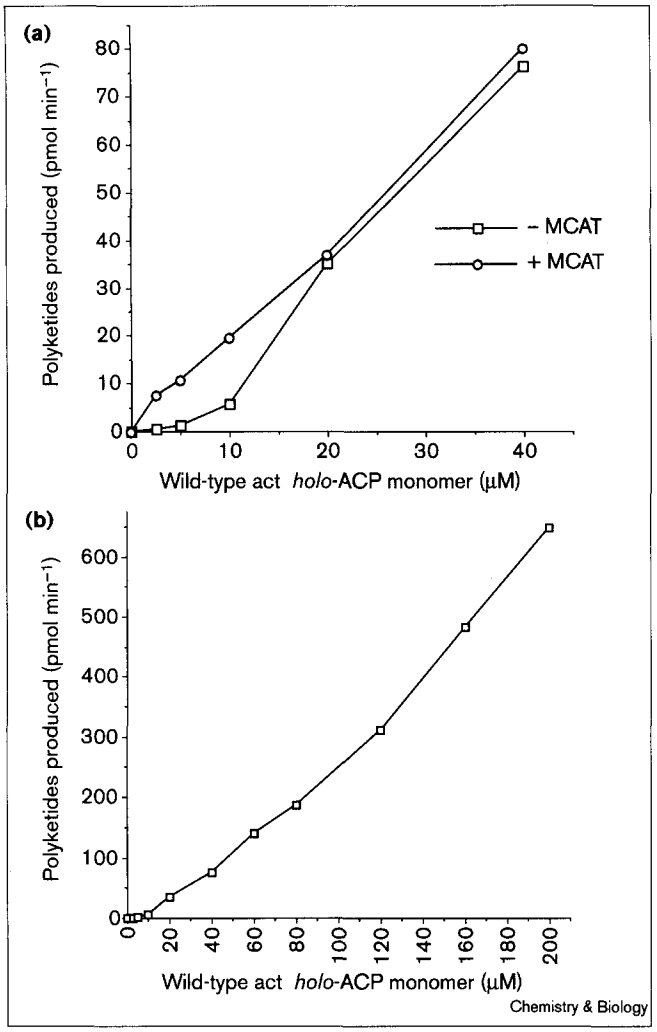
MCAT does not operate as a thiolesterase

A further difference between the type II PKSs and the modular PKSs is the apparent lack of a thiolesterase homologue in the gene clusters. One potential role for MCAT could be to act as a thiolesterase, removing the fully formed polyketide from the ACP. We tested this possibility using preformed malonyl ACP. This was achieved using our previously reported method of simply incubating the C17S act *holo*-ACP monomer with malonyl CoA. Malonyl-ACP was repurified and the acylated protein incubated with KS/CLF for 2 h at 30°C. Both SEK4 and SEK4b were formed. In a 100 μl assay containing malonyl C17S act ACP (14.6 μM) a total of 191 pmol of SEK4 and SEK4b were produced (average of two runs). This corresponds to a quantitative yield of octaketides as the assay contained 1.46 nmol (that is 8 × 182 pmol) of available malonyl groups.

Discussion

We set out to study the activity of the act minimal PKS *in vitro* using rigorously purified proteins. The protein expression strategy described previously [5,6] for production of

Figure 8



Variation in the rate of polyketide production (SEK4 and SEK4b) with ACP concentration. **(a)** Variation in the rate in the presence or absence of His_6-MCAT (1 μM) at lower ACP concentrations. **(b)** Effect of increasing ACP concentration in the absence of His_6-MCAT.

act KS/CLF involved the cloning of type II PKS genes using the *act*II ORF4 promotor system in low copy number vectors such as pSEK38. Hutchinson and coworkers [21] have used the *ermEp** promoter in a similar way for expressing the tcm genes. We decided to utilise the thiostrepton induction system devised for *Streptomyces* by Bibb and coworkers [29]. This system provides a convenient method of induction by the *tip*A promoter and provides large amounts of soluble protein. Additionally, the fusing of a His_6 tag at the amino terminus sequence allowed rapid initial purification using Ni^{2+} affinity chromatography.

We realised that potential problems could be experienced in purifying expressed KS/CLF/ACP complexes. Because there is no published data on the dissociation constants for components of these complexes, we decided to express the KS and CLF components in the absence of ACP, and then add our previously purified ACPs. This strategy differs from that used by Carreras and Khosla [23], who described the removal of ACP from expressed KS/CLF/ACP complexes by repeated chromatography. Our attempts to repeat this work using KS/CLF/ACP as expressed from pSEK4 in *S. coelicolor* CH999 showed that ACP could not be removed completely under standard chromatographic conditions (data not shown) and that even extensively purified extracts displayed PKS activity without the requirement for additional ACP (data not shown). Our strategy allows us to rigorously rule out the possibility of bound act ACP, enabling us to titrate the amount of added ACP. Furthermore, it allows us to probe the role of ACP by supplementing the *in vitro* minimal PKSs with a variety of heterologous and mutant ACPs.

Our previous work with wild-type act ACP showed that expression from *E. coli* leads to production of predominantly (> 90%) inactive *apo*-protein lacking the 4′-phosphopantetheine prosthetic group [25]. We have been able to produce high levels of type II PKS *holo*-ACPs by coexpressing the ACP with the *E. coli* ACPS such that act ACP is present in predominantly (> 95%) *holo* form [26]. Additionally we have shown that for wild-type act ACP, formation of an internal disulphide between the 4′-phosphopantetheine terminal thiol and Cys17 is facile and rapid [27]. Even in C17S mutants act *holo*-ACP rapidly dimerises through disulphide formation between the phosphopantetheine thiols at low DTT concentrations (1.0 mM or less) and that monomerisation by DTT is relatively slow (Figure 2a). Because act *holo*-ACP monomer is the only active species of ACP for polyketide production it is important to assess the levels of *holo*-ACP monomer added to PKS assays using ESMS or native PAGE, for example (Figure 2). It is also important to estimate the likely concentrations of *holo*-ACP monomer used in published studies for comparison with the work reported here (see below). Act *holo*-ACP itself was expressed in *E. coli* and purified to homogeneity. Care was taken at all times to monitor the ratio of active act *holo*-ACP monomer versus inactive disulphide dimer using ESMS and native PAGE (Figure 2). Addition of at least 2 mM DTT was required at all times during purification and *holo*-ACP was pre-incubated with DTT at 2 mM before polyketide production assays if necessary. In this way we could be confident that the ACP component of the assay was fully active.

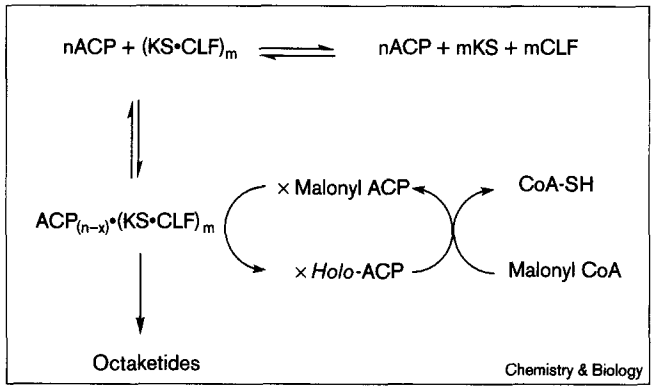
Initial experiments were consistent with the observations of Hutchinson and coworkers [24]. Reconstitution of act KS/CLF at 1.5 μM in the presence of 2.5 μM act *holo*-ACP monomer produced a mixture of the expected SEK4 and SEK4b metabolites at a rate of around 1 pmol min^{-1} (Figure 8a) in the presence of 1 mM malonyl CoA. This rate was substantially increased to around 8 pmol min^{-1}

when 1 μM MCAT was added. An increase in act *holo*-ACP monomer concentration to 10 μM similarly increased the rate of polyketide production in the absence of MCAT, however. Indeed, increasing the concentration of act *holo*-ACP monomer increased the rate of polyketide production in a near linear fashion at concentrations above 10 μM. No significant curvature in the plot of polyketide production rate versus act *holo*-ACP concentration was observed at ACP concentrations up to 200 μM (Figure 8b). At the highest concentrations of ACP tested in these experiments polyketides were produced at a rate of approximately 650 pmol min^{-1}. Calculation of the rate of *holo*-ACP self-malonylation under these conditions (using the k_{cat} value of 0.34 min^{-1} determined previously) [22] indicates a total malonyl ACP generation rate of 6800 pmol min^{-1} (or, divided by eight, 850 pmol min^{-1} of octaketides). This shows that the rate of octaketide production is limited by the rate of malonyl ACP production. The effect of additional MCAT was marginal at ACP concentrations between 10 and 20 μM, and was undetectable at concentrations above 20 μM ACP.

At low ACP concentrations the rate of polyketide production appeared to be depressed below the value expected from a linear relationship between the *holo*-ACP monomer concentration and the rate of polyketide production. In order to test whether this was due to the absolute concentration of *holo*-ACP monomer or whether it was due to the ratio of ACP:KS/CLF we conducted assays containing equimolar amounts of ACP:KS/CLF at 10 μM and 20 μM. The polyketide production rate was significantly diminished as compared with the same ACP concentrations with 1.5 μM KS/CLF. The effect of total protein concentration was determined by the addition of BSA (80 μM) to a standard assay. In this case a stimulation of the rate of polyketide production was observed. Clearly the ratio of *holo*-ACP monomer: KS/CLF is crucial in determining the rate of polyketide production — *holo*-ACP must be present in excess over KS/CLF.

The observed depression of polyketide synthesis at low *holo*-ACP monomer concentrations could be the result of a number of factors. One possibility is that the assays are conducted at a concentration where there is insufficient association between KS, CLF and ACP to form a productive complex. Addition of ACP might shift this equilibrium (Figure 9) allowing more productive complex to be formed, and thus increasing the rate of polyketide production, as we observed. If this were the case then an increase in KS/CLF concentration should also increase polyketide production. Increasing the KS/CLF concentration, however, dramatically attenuates polyketide production. There might be a small general stimulation of polyketide production due to overall protein concentration, but this is clearly insufficient to explain the observed nonlinear rate dependence on ACP at lower concentrations.

Figure 9



Scheme indicating possible sequestration of ACP by the KS/CLF complex. The ratio (n-x)/m is unknown. Please see text for more details.

It does not seem unreasonable to speculate that KS/CLF will bind *holo*-ACP, thus reducing its free concentration in solution. This could thereby reduce the rate of *holo*-ACP self-malonylation at ACP concentrations below the stoichiometric amount of ACP (whether this be 1:1, 2:1 or another ratio). Our results are consistent with the sequestering of *holo*-ACP from solution. At concentrations above the stoichiometric amount of ACP, the excess free ACP is able to undergo self-malonylation and there is a consequent increase in the rate of polyketide production as malonyl ACP becomes available for the synthase complex. A major difficulty in interpreting the data presented here is caused by the methods used for quantifying the individual proteins. *Holo*-ACP was quantified by weighing freeze-dried desalted samples. Although, to the best of our knowledge, there is minimal remaining salt, it is difficult to be precise and ACP concentrations are likely to be an overestimate. KS/CLF concentrations were determined by the method of Bradford, using BSA as a standard. There are many reports of proteins behaving anomalously in Bradford assays and the actual concentrations used here could be different [30]. For this reason it is not possible to use the data presented here to determine an exact stoichiometry of KS/CLF/ACP, but it is clear from our results that the KS/CLF must bind at least one equivalent of ACP before synthesis begins efficiently.

Our results indicate a requirement for MCAT only at *holo*-ACP concentrations that are in the region of the KS/CLF concentration. This could be the result of a number of factors. One possibility is that MCAT transfers malonyl groups from malonyl CoA to bound ACP more effectively. Alternatively, MCAT might simply increase the rate of malonyl transfer to free *holo*-ACP. At substoichiometric concentrations of ACP, where most of the ACP will be bound, the free *holo*-ACP concentration will be very low and the self-malonylation rate depressed. An alternative possibility is that the MCAT aids turnover by unblocking

stalled complexes in which the ACP thiol is blocked by an acetyl group. Decarboxylation of malonyl ACP has been invoked as a possible source of the acetyl starter unit [24]. The resulting acetyl group is then transferred to the free thiol of the KS. If the decarboxylation took place with significant frequency (perhaps as a side reaction) when the KS active site was already loaded with a partially formed chain, the set of proteins would be at a dead end with no turnover possible until one of the thiols was free. We have shown, however, that MCAT has no measurable acetyltransferase activity (T.S Hitchman, R.J.C., J.C and T.J.S, unpublished observations) and so it might not be very effective in removing the acetyl group from ACP.

Comparison of these results with precedents in the literature

In contrast to previous reports, we have demonstrated that MCAT is not essential for polyketide biosynthesis in *in vitro* reconstituted minimal PKS reactions. Our results show that the reaction proceeds at a rate governed by the rate of ACP self-malonylation. At low ACP concentrations MCAT does, however, stimulate an increase in the rate of polyketide production. In contrast, Carreras and Khosla [23] have reported that self-malonylation of ACP is not observed in their system and that MCAT is an absolute requirement for polyketide production. They do report observation of some apparent autocatalytic malonylation of *holo*-ACP but have ascribed this to the use of an impure protein preparation, as the effect was not found on further purification of the ACP. Hutchinson and coworkers [24] report a low level of self-malonylation that would apparently account for a maximum of 10% of polyketide production in the tcm *in vitro* minimal system. These observations appear to reinforce the earlier reports of Revill and Hutchinson and coworkers [19,20] in which they did not observe ACP self-malonylation in MCAT assays.

In order to explain these differences it is important to examine the reactivity of type II PKS ACPs used in each study. We have shown that *holo*-ACPs bind acyl groups (and presumably growing polyketide chains) as thiol esters to the terminal thiol of the phosphopantetheine prosthetic group [22]. The first prerequisite for ACP activity is that it be present in the *holo* form. *E. coli* expression of type II PKS ACPs leads to production of predominantly (sometimes exclusively) *apo*-ACP as the ACPs are poor substrates for the endogenous *E. coli* ACPS. The second prerequisite is that the phosphopantetheine thiol is available for reaction. For wild-type act *holo*-ACP, at least, the terminal phosphopantetheine thiol can rapidly form an internal disulphide with Cys17, or it can dimerise with another ACP unit. As our results show, monomerisation of the dimers is slow at 1 mM DTT. Dimer formation is a feature of all purification protocols we have examined, and it is particularly rapid during freeze-drying or sephadex-desalting procedures. Addition of ACP dimers to PKS assays containing DTT would only slowly release free active *holo*-ACP monomer, resulting in slow polyketide formation (Figure 6). For this reason it is crucial to examine the *holo*-ACPs by ESMS or native PAGE before use in PKS assays. Furthermore the concentration of malonyl CoA should be considered. We have measured a K_M for self-malonylation of act ACP as ~200 μM, and for reactions to proceed with an appreciable rate, the malonyl CoA concentration should be in this region [22].

The MCAT experiments of Revill [19], utilising act ACP expressed from *E. coli*, used total act ACP concentrations of ~40 μM, but only 1–2% of the 40 μM was *holo*-ACP. Thus, the concentrations of *holo*-ACP in this assay would likely have been 0.4–0.8 μM, which would have been further reduced if significant amounts of internal disulphide with Cys17 or disulphide dimers were present (as there would be after the lyophilisation step of the purification procedure). The malonyl CoA concentration was 23 μM. It is clear from our previous results that ACP self-malonylation would be very slow at these substrate concentrations. Here we have also shown that MCAT activity is most obvious at these low *holo*-ACP monomer concentrations. Under these conditions, therefore, it would have been easy to overlook ACP self-malonylation. The MCAT assays reported by Hutchinson and coworkers [20] used tcm ACP isolated from *S. glaucescens*, which was presumably present as *holo* protein. No indication of the amounts of internal disulphide (Cys16 of tcm ACP is equivalent to the Cys17 of act ACP) or disulphide dimers was given and so it is possible that the concentration of active tcm *holo*-ACP monomer present in the assays was below the 25 μM total ACP concentration. The malonyl CoA concentration in these assays was 5 μM and self-acylation of tcm ACP would be difficult to observe under these conditions.

The *in vitro* experiments described by Carreras and Khosla [23] using purified components to reconstruct heterologous type II PKS systems used fren ACP expressed in *E. coli*. The purified *apo*-ACP was treated with purified *E. coli* ACPS and CoA to produce *holo*-ACP. Although earlier reports claimed up to 95% *holo*-protein, evidence for the ratio of active *holo*-ACP monomers to inactive dimer has not been presented. The assays reported by this group have used apparent ACP concentrations of 10 μM, although the concentration of active *holo*-ACP monomer could well have been lower. Again, under these conditions MCAT does stimulate transfer of malonyl groups from CoA to *holo*-ACP, whereas self-acylation is relatively slow. It is perhaps worthy to note that Carreras and Khosla [23] did not use the homologous wild-type act ACP, which we now know forms a stable intramolecular disulphide, in any of their reported experiments.

Interestingly Khosla, Walsh and coworkers [4] have carried out assays where self-malonylation of ACP might have been

expected to provide both the starter and extender units required for polyketide production. In these experiments ^{14}C-acetyl fren ACP was produced by incubating fren *apo*-ACP with ^{14}C-acetyl CoA and purified *E. coli* ACPS in a reaction similar to that previously described by us [26]. This provided ^{14}C-acetyl fren ACP in a form that could not include any inactive dimeric protein. PKS assays were then conducted that contained 125 μM ^{14}C-acetyl fren ACP, 1 mM malonyl CoA and ~1 μM act KS/CLF. Although no MCAT was added it might have been assumed to be a component of the semi-pure KS/CLF preparation. The labelled acetyl groups initiated polyketide synthesis, and the fren *holo*-ACP would have been released at the end of each round of octaketide production. ^{14}C-Labelled product was quantified and shown to correspond to a quantitative yield of polyketides. Any unlabelled polyketides arising from reactions of the free fren *holo*-ACP monomer, such as we have described, would not have been observed by this method as the malonyl extender units were unlabelled.

The experiments reported recently by Hutchinson and coworkers [24] have used tcm ACP obtained from *S. glaucescens* as *holo*-protein. They observed slow self-acylation of ACP. The conditions reported for this reaction, however, utilised a very low concentration of malonyl CoA (2 μM) compared with the 150 μM malonyl CoA typically used in their polyketide production assays. Because the K_M for malonyl CoA for act ACP self-malonylation is around 200 μM, self acylation would certainly be expected to be slow under these conditions. Our PKS assays, in common with those of Carreras and Khosla [23], use a malonyl CoA concentration of 1mM.

We have previously shown that our *holo*-ACP monomer preparations are free from *E. coli* MCAT [22]. We believed it was necessary to rigorously exclude the possibility of contaminating *S. coelicolor* MCAT in the KS/CLF preparation. The presence of MCAT seems unlikely as addition of the *S. coelicolor* His_6-MCAT to 1 μM (i.e. approximately equimolar with KS/CLF) is required for the observed stimulation of polyketide production. Presence of MCAT at this level would be clearly visible in the SDS PAGE examination of the KS/CLF preparation. Furthermore, KS/CLF preparations were inactive in assays to measure the transfer of malonyl groups from ^{14}C-malonyl CoA to *E. coli* FAS ACP, which we have shown previously is a good substrate for *S. coelicolor* MCAT (T.S. Hitchman, R.J.C., J.C. and T.J.S, unpublished observations). At best the KS/CLF preparation used here could generate malonyl ACP at around 15 nM min^{-1}, a rate insufficient for the observed rapid production of polyketides (~8000 nM min^{-1} is required under similar conditions).

Requirement for MCAT

MCAT is clearly not required for polyketide biosynthesis *in vitro* when the concentration of act *holo*-ACP monomer is appreciably larger than the KS/CLF concentration. At lower ACP concentrations the effect of additional MCAT is an increase of the rate of polyketide production. Under these conditions the free *holo*-ACP monomer concentration is likely to be low because of sequestration by KS/CLF and self-acylation is depressed. It might be that MCAT is efficient at stimulating malonyl transfer to very low concentrations of act *holo*-ACP monomer, or that it can efficiently transfer malonyl groups to bound *holo*-ACP. The *in vivo* concentrations of MCAT, KS/CLF, ACP and malonyl CoA are unknown and it is not possible to determine the requirement for MCAT *in vivo*.

Starter unit

Our assays contained no exogenous acetyl CoA, although commercial sources of malonyl CoA are known to contain acetyl CoA at low levels. Our previous results have shown that neither MCAT nor act *holo*-ACP can catalyse the transfer of acetyl groups from CoA onto *holo*-ACP. Because the acetyl group is required by the synthase as a 'starter unit' it must be derived using one or other of two mechanisms. The act KS/CLF might contain an acetyl-transferase activity that could utilise acetyl CoA impurities in the malonyl CoA. This would seem unlikely given the results of Sherman and coworkers [14] which show that the putative AT domain of KS is unnecessary for act biosynthesis *in vivo*. Alternatively, we have shown here that KS/CLF in the presence of purified malonyl ACP produces exactly the expected amount of octaketides. Secondly, decarboxylation of malonyl ACP could be catalysed by KS/CLF. We have shown previously that act malonyl ACP can catalyse decarboxylation of the malonyl group, but only under conditions of partial denaturation [22]. Thus efficient production of acetyl ACP would need to be catalysed by a separate entity. The point at which this decarboxylation could take place is uncertain, as there is no mechanistic requirement for the terminal carboxyl group to be lost before subsequent elongation events. Analogy with FAS would suggest production of acetyl ACP as a starter unit. Acetyl ACP, derived by treatment of *apo*-ACP with acetyl CoA and ACPS, has been shown to be capable of initiating polyketide biosynthesis *in vitro* by Carreras *et al.* [5]. It is also conceivable that decarboxylation could also be a later-stage event. This could be a clue to understanding the biosynthesis of oxytetracycline where malonyl or malonoamyl groups appear to be the starter unit in polyketide biosynthesis.

Rate of polyketide production

Our results show that for the reconstituted homologous act type II PKS, which consists of KS, CLF and ACP, the rate of polyketide production depends upon the concentration of the act *holo*-ACP monomer. Act *holo*-ACP disulphide dimer only releases active ACP monomer slowly on reaction with 1 mM DTT, and polyketide production by the KS/CLF/ACP complex was depressed well below the level observed for active *holo*-ACP monomer. Neither *E. coli* FAS

holo-ACP nor act *apo*-ACP is active in the reaction. The limiting step in polyketide synthesis for the act KS/CLF/ACP complex seems to be the transfer of malonate from CoA to ACP. This is clearly not possible for assays utilising either *apo*-ACP or FAS ACP. The overall reaction is not dependent on *S. coelicolor* MCAT *in vitro*.

Significance

Studies on the mechanism of polyketide chain assembly catalysed by type II polyketide synthases (PKSs) require development of *in vitro* systems comprising the minimal components of the PKS complex necessary for *de novo* polyketide synthesis. *In vivo* studies indicated that the minimal system comprised the ketosynthase (KS) and 'chain length factor' (CLF) along with the acyl carrier protein (ACP), whereas recent *in vitro* studies suggested an additional requirement for malonyl CoA:ACP transacylase (MCAT) to catalyse transfer of malonate from malonyl CoA to the ACP. This latter result contrasted with studies which had demonstrated that type II PKS *holo*-ACPs were capable of self-malonylation. This apparent contradiction has been resolved and exact conditions for *in vitro* polyketide synthesis have been defined. The concentration of *holo* ACP monomer appears to be rate-limiting for the production of polyketides by the minimal act PKS. The overall rate of polyketide production is of the order of magnitude expected if self-acylation of the ACP is the slowest step during polyketide biosynthesis. These observations are clearly supported by the *in vivo* experiments of Decker *et al.* [31] who showed that the level of tetracenomycin (tcm) production is enhanced when the tcm ACP is overexpressed in *S. glaucescens*. The accurate measurement of kinetic constants for MCAT activity and ACP self-acylation leads us no closer to an understanding of the *in vivo* conditions leading to actinorhodin synthesis in *S. coelicolor*. The significance of these *in vitro* measurements to the *in vivo* situation await the determination of *in vivo* protein and substrate concentrations. We have determined, however, that the rate of polyketide production depends on the ratio of KS/CLF to ACP. MCAT might be required for efficient polyketide production *in vivo* if the free *holo*-ACP concentration is low, but the synthase can operate without the aid of MCAT.

The self-acylation mechanism could represent a fossil activity of the act PKS. The clustering of the genes for act biosynthesis (and very many other polyketide secondary metabolites), together with appropriate resistance genes, could be a relic of gene dispersion events in the past. For a set of genes to be preserved by an organism they should confer a selective advantage (e.g. production of, and self-resistance to, a useful antibiotic). A gene cluster transferred from one heterologous organism to another that was unable to initiate its synthesis because of incompatibility with the host's MCAT would not, presumably, confer any evolutionary advantage on the organism. A protein that was able to obtain a malonyl group from its host, either through self-acylation or through adventitious interaction with MCAT, would be able to produce polyketides and become useful to the organism. A self-acylation mechanism would be a clear initial advantage. Later evolution could lead to enhanced polyketide production if the ACP were able to better interact with the host's fatty acid synthase (FAS) MCAT. The use of a separate MCAT by FAS systems might be a reflection of the additional control processes required for the crucial primary metabolism of fatty acids.

The demonstration of the true minimal requirements for polyketide synthesis *in vitro* should facilitate further *in vitro* studies to clarify the exact role of each PKS component and the exact mechanisms of polyketide chain assembly, cyclisation and reductive modifications. This, in turn, should allow a more complete assessment to be made of the real potential for genetic manipulations of type II PKSs to provide novel compounds by rational design.

Materials and methods

Cloning, expression and purification of actI ORFs 1 and 2 (KS and CLF)

Standard procedures were adopted unless otherwise indicated [32,33]. A pBluescript vector containing an 8.8 kb fragment of the act cluster between *Pst*I-12 and *Pst*I-20 [34] was digested with *Sac*I. The vector and a 2.82 kb *Sac*I fragment were gel purified [35]. The vector, now containing a 1.89 kb genomic fragment including the first 205 base pairs of actI ORF 1 was recloned with T4 DNA ligase and cloned into *E. coli* DH5α. The cloned vector was used as a PCR template using the pBluescript reverse primer and a synthetic oligonucleotide 5′-CATATGAAGCGCAGAGTCGTCATCAC-3′ containing the sequence for *Nde*I (5′-CATATG-3′) overlapping the first codon. The primers were phosphorylated before use. The PCR conditions were: 96°C for 1 min, then 64°C for 45 s, then 72°C for 2 min, repeated 25 times. Vent DNA polymerase (NEB) was used, together with a Mg^{2+} concentration of 4 mM and other conditions as specified by the suppliers. A blunt-ended product of the expected 220 base pairs was gel purified and ligated into the dephosphorylated *Sma*I site of pBluescript. After transformation into *E. coli* DH5α the sequence was checked by manual sequencing (Promega). One positive clone that contained no unwanted mutations was then linearised with *Sac*I and the 2.82 kilobase *Sac*I fragment containing the rest of *act*I ORF1, and complete ORFs 2 and 3 was ligated into this. After transformation into *E. coli* DH5α the direction of cloning was checked by restriction analysis. One positive clone was designated as pRJCII04F and was used for further manipulations.

For construction of an expression clone containing only KS and CLF, pRJCII04F was digested with *Nde*I and *Nco*I. The resulting 2.5 kilobase fragment was ligated between the *Nde*I and *Nco*I sites of pLitmus28 (NEB) and re-excised as a *Nde*I–*Eco*RI fragment. This was then ligated between the *Nde*I and *Eco*RI sites of pIJ4122 followed by transformation into *S. lividans*. Positive clones were selected by PCR analysis of inserts directly from lysed protoplasts. One positive clone, designated pRJC006, was transformed into *S. coelicolor* CH999.

Spores of *S. coelicolor* CH999 pRJC006 were grown in 5 ml of Super YEME containing kanamycin 50 μg/ml for 3 days at 30°C shaking at 250 rpm. The mycelia were then transferred to 50ml of Super YEME containing kanamycin at 50 μg/ml and grown as before for a further

48 h. Induction was performed by addition of thiostrepton to 5 μg/ml and growth was continued as before for another 24 h. Cells were harvested by centrifugation and resuspended in 40 ml of 0.15 M KH_2PO_4 pH 7.5 containing 10% glycerol and frozen. A cell-free protein extract was prepared according to the method of Carreras *et al.* [6] and then the buffer exchanged by sepharose chromatography to 0.1 M KH_2PO_4, 0.5 M NaCl, 10% glycerol, pH7.5. The entire extract was loaded onto a nickel affinity column (Novagen, 0.5 ml). The column was eluted with 0.1 M KH_2PO_4, 0.5M NaCl, 10% glycerol buffer containing increasing amounts of imidazole: 0.0 mM, 2 ml; 1.0 mM, 1.5 ml; 2.5 mM, 1.5 ml; 5 mM, 1.5 ml; 10 mM, 1 ml; 20 mM, 1 ml; 50 mM, 1 ml; 500 mM, 1 ml. Fractions containing 50 mM and 500 mM imidazole contained the required protein (SDS PAGE), but the 500 mM cut was of significantly higher purity. The 500 mM fraction was desalted into 0.1 M KH_2PO_4, 2 mM DTT, 2 mM EDTA, 10% glycerol at pH 7.3 to a total volume of 2.2 ml. Bradford protein assay indicated protein was present at 0.69 mg/ml. This protein fraction was used for all further assays.

Expression, purification and acylation of act I ORF3 (ACP)

Expression and purification of *holo*-ACPs was as described previously [25,26]. Disulphide dimers were prepared by resuspension of freeze-dried purified *holo*-ACPs in 100 mM phosphate buffer pH 7.5 containing no DTT, or by desalting using a sepharose column and buffers not containing DTT. Purified ACP dimer was analysed by native PAGE and ESMS. Malonylation of ACP was achieved by incubation of *holo*-ACP monomer (43 μM) with malonyl CoA (1.2 mM) for 30 min in phosphate buffer pH 7.3 containing 2.0 mM DTT at 30°C. Malonyl ACP was purified by desalting against phosphate buffer pH 7.3 without DTT using sepharose chromatography. ACP was quantified by weighing desalted freeze-dried purified protein.

Expression and purification of fabD *(MCAT)*

His_6-MCAT was expressed and purified from *E. coli* as described previously (T.S Hitchman, R.J.C., J.C. and T.J.S, unpublished observations).

Determination of MCAT activity of purified KS/CLF fraction

E. coli FAS ACP (Sigma) was purified according to our previously reported methods [25]. Reactions contained ^{14}C-malonyl CoA (1.7 GBq $mmol^{-1}$, 22 μM) and as appropriate ACP (50 μM) and/or KS/CLF (2.5 μM) made up to a total volume of 10 μl with 50 mM phosphate buffer, pH 6.5 containing 2 mM DTT. Assays were conducted for 30 min at 30°C, then were quenched by addition of BSA (30 μl, 100 mg/ml) and TCA (30 μl, 50%). Protein was precipitated at 0°C for 10 min, then pelleted (13000 rpm, 10 min). The supernatant was discarded and the pellet washed with 10% ice cold TCA. The pellet was resuspended in 2 N NaOH (50 μl) and 2 N Tris (50 μl) and the solution counted. Reactions and controls were performed in triplicate.

Polyketide synthase assay and HPLC analysis

SEK4 and SEK4b were purified from a culture of *S. coelicolor* CH999 pSEK4 (gift of Chaitan Khosla) grown on solid R5 media [34] for 7 days at 30°C. 60 Plates were diced and extracted with a solution of ethyl acetate and methanol (4:1, 3 × 1l). The organic extracts were dried ($MgSO_4$), evaporated to dryness and the brown residue mixed with ethyl acetate (200 ml). The resulting suspension was filtered and the filtrate evaporated *in vacuo*. The solid residue was dissolved in the minimum amount of methanol at room temperature. The solution was cooled to −20°C overnight and the precipitate of crude SEK4b (200 mg) collected by filtration as a pale cream powder. The supernatant was evaporated to dryness and dissolved in the minimum amount of ethyl acetate and then purified by flash chromatography according to the method of Still *et al.* [36]. Crude SEK4 was eluted with acetonitrile and obtained as a brown powder (60 mg). SEK4 and SEK4b were purified by RP HPLC using the following method. Solvent A 0.05% trifluoroacetic acid in water; solvent B 0.045% trifluoroacetic acid in acetonitrile; Column Rainin Microsorb C_{18} 30 × 1 cm eluted at 4 ml/min, observed simultaneously at 254 nm and 280 nm. Injections of approximately 1 mg of either crude SEK4 or crude SEK4b were made and the column eluted for 5 min at 0% B. A gradient to 75% B was then made over 30 min. SEK4 was eluted at 26 min after injection, SEK4b after 28 min. ^{1}H NMR analyses for each compound agreed with published data. Purified compounds were used to calibrate further HPLC analysis.

Polyketide synthase assays were performed at 30°C for between 5 and 20 min in 100 mM phosphate buffer, pH 7.3 containing 2 mM DTT and 2 mM EDTA in a total final volume of 100 μl. After incubation the assays were quenched by addition of 100 mg of solid NaH_2PO_4, then extracted with ethyl acetate (2 × 500 μl). The organic extract was evaporated and the residue dissolved in methanol (130 μl). 59 μl of the extract was injected onto a Luna(2) C_{18} RP-column 250 × 4.6 mm (Phenomenex). The column was eluted at 1 ml/min for 5 min at 0%B. A gradient to 75%B was then made over 30 min. SEK4 was eluted at approx 21 min after injection, SEK4b after approx 22 min.

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