

Catalytic self-acylation of type II polyketide synthase acyl carrier proteins

Timothy S Hitchman, John Crosby, Kate J Byrom, Russell J Cox and Thomas J Simpson

Background: Aromatic polyketides are synthesised in streptomycetes by the successive condensation of simple carboxylic acids, catalysed by multienzyme complexes – the polyketide synthases (PKSs). Polyketide assembly intermediates are covalently linked as thioesters to the holo-acyl carrier protein (ACP) subunit of these type II PKSs. The ACP is primed for chain elongation by the transfer of malonate from malonyl CoA. Malonylation of fatty acid synthase (FAS) ACPs is catalysed by specific malonyl transferase (MT) enzymes. The type II PKS gene clusters apparently lack genes encoding such MT proteins, however. It has been proposed that the MT subunit of the FAS in streptomycetes catalyses malonylation of both FAS and PKS ACPs *in vivo*.

Results: We demonstrate that type II PKS ACPs catalyse self-malonylation upon incubation with malonyl CoA *in vitro*. The self-malonylation reaction of the actinorhodin C17S holo-ACP has a K_m for malonyl CoA of 219 μM and a k_{cat} of 0.34 min^{-1} . Complete acylation of the PKS ACPs was observed with malonyl, methylmalonyl and acetoacetyl CoAs. No reaction was observed with acetyl and butyryl CoAs and FAS ACPs did not react with any of the substrates. Recombinant FAS MT from *Streptomyces coelicolor* did not accelerate the rate of malonylation.

Conclusions: The catalytic self-acylation of type II PKS ACPs is an unprecedented reaction. We propose a reaction mechanism in which conserved arginines form a salt bridge with the acyl moiety and sequester it from bulk solvent. This work suggests that the β -ketoacyl synthase, chain length factor and ACP may constitute a truly minimal PKS *in vivo*.

Introduction

Polyketide metabolites isolated from bacteria, fungi and plants have diverse biological activities and provide an important source of new pharmaceutical and agrochemical agents (Figure 1) [1]. Recent advances in our understanding of polyketide biosynthesis have made the generation of novel bioactive compounds by genetic manipulation a realisable goal [2,3]. Polyketide chain assembly is catalysed by multifunctional polyketide synthase (PKS) enzyme complexes that are structurally and functionally analogous to fatty acid synthases (FASs; Figure 2) [4]. PKS-mediated condensation of acyl thioesters results in the formation of a β -keto moiety. The subsequent sequence of ketoreduction, dehydration and enoylreduction normally prevalent in fatty acid biosynthesis may be partly or fully omitted by the PKS in each assembly cycle in a highly programmed manner. This programming is the key to the structural and chemical diversity observed in polyketide natural products.

Polyketide assembly

The programming of the assembly sequence in polyketide biosynthesis is achieved by arranging a similar set

of enzyme activities in either of two ways. The modular type I PKSs, which catalyse the biosynthesis of reduced macrolide polyketides such as erythromycin, comprise large multifunctional proteins [5]. Each reaction of the pathway is catalysed by a unique active site and the enzyme domains are assembled into linear modules so that each module contains all the active sites necessary for one cycle of elongation and reduction; there is one module present per chain extension. In contrast, the aromatic polyketides are assembled by the iterative use of a single set of active sites. The active sites may be contained on a single polypeptide (type I), as in 6-methylsalicylic acid synthase (MSAS) [6], or they may be a complex of small, discrete, monofunctional proteins (type II), such as the actinorhodin (act) PKS [1,7,8]. The genes encoding these enzymes are clustered, thus the entire set of genes necessary for the type II act PKS is contained within a single 5.7 kilobase (kb) segment of the *Streptomyces coelicolor* chromosome [7–9]. Although significant progress in understanding the empirical rules governing aromatic polyketide assembly has been made using genetic manipulation *in vivo* [10], the mechanism of programming

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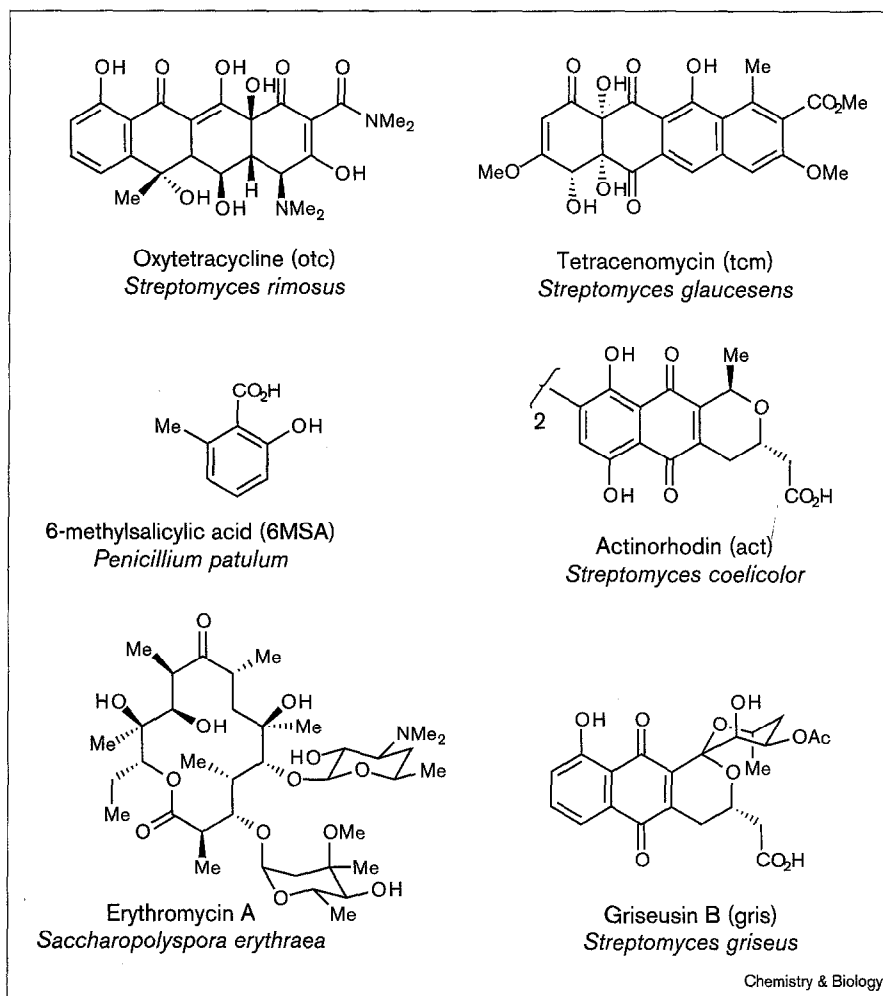
Key words: acyl carrier protein, fatty acid synthase, malonyl transferase, polyketide synthase

Received: 15 September 1997
Revisions requested: 10 October 1997
Revisions received: 4 November 1997
Accepted: 18 November 1997

Published: 15 January 1998
Chemistry & Biology January 1998, 5:35–47
<http://biomednet.com/elecref/1074552100500035>

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Figure 1



The structures of several polyketide metabolites and their sources.

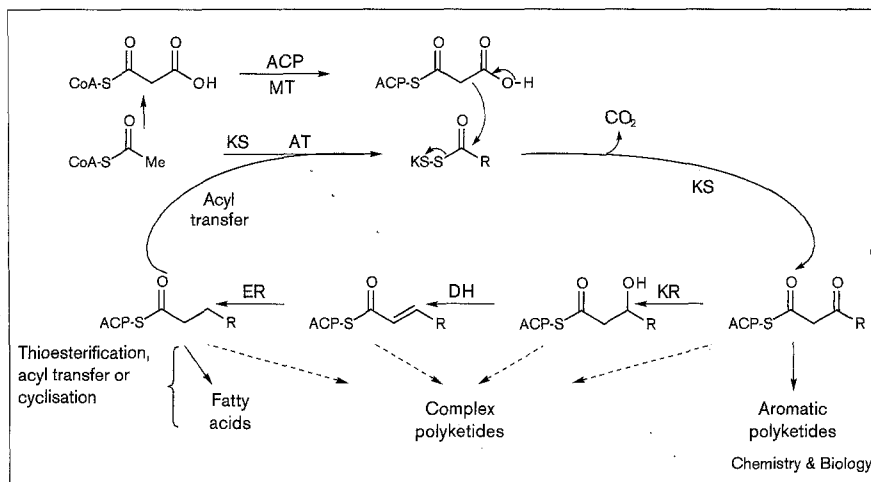
in these cases is obscure and elucidation of the structural and enzymatic details requires a precise understanding of the biochemistry of the purified proteins.

Early studies on polyketide biosynthesis were greatly facilitated by previous studies on the fatty acid biosynthetic pathway, which is both chemically and architecturally analogous. Thus, the type II FAS from *Escherichia coli* has provided a useful model for the study of bacterial PKS multienzyme complexes. Despite the similarities in both sequence and structure of the component proteins there are major differences in the requirements of the pathways. The *E. coli* FAS complex catalyses the full reduction of each β -keto moiety prior to further chain extension. In contrast, the type II PKSs catalyse the assembly of a highly oxidised carbon chain, in which little or no reduction has occurred. The *E. coli* FAS proteins must therefore interact with both polar and hydrophobic groups, whereas the PKS subunits must stabilise a highly reactive poly- β -keto chain and prevent incorrect and

spontaneous cyclisation. Recent genetic experiments have facilitated the partial characterisation of many of the type II PKS subunits *in vivo*, and have provided a model for polyketide assembly [10]. In this model a starter acyl unit, normally acetate, is transferred from coenzyme A (CoA) onto the active site thiol of the β -ketoacyl synthase (KS) and a malonate extender unit is transferred from CoA onto the pantetheine thiol of the holo form of the acyl carrier protein (ACP). The KS then catalyses a decarboxylative Claisen condensation between the two acyl units to generate acetoacetyl ACP. Successive addition of extender units results in the assembly of a highly reactive poly- β -keto chain, the length of which is thought to be determined, at least in part, by the cooperative action of the KS with the so-called chain length factor (CLF) [11]. Regiospecific ketoreduction, cyclisation and aromatisation are then catalysed, where required, by additional specific enzymes later in the pathway. Genetic manipulation of the KS, CLF and ACP PKS subunits *in vivo* has demonstrated that, in the absence of later enzymes, the

Figure 2

A generalised scheme of fatty acid and polyketide biosynthesis [1,4]. The assembly of fatty acids is catalysed by a series of enzyme activities in a highly specific manner. The FAS/PKS complex is primed when the acetyl transferase (AT) acetylates the ketosynthase (KS) and malonyl transferase (MT) catalyses loading of the malonyl extender unit from malonyl CoA to the pantetheine thiol of the acyl carrier protein (ACP). After chain extension catalysed by KS, the β -carbonyl is removed by sequential action of a ketoreductase (KR), dehydratase (DH) and enoylreductase (ER). In fatty acid biosynthesis full reduction always occurs, whereas in polyketide biosynthesis full, partial or no reduction occurs in a programmed manner. After a defined number of elongation/reduction cycles the nascent chain is released from the FAS/PKS complex by thioesterification, acyl transfer or cyclisation. The choices of starter and



extender units, degree of reduction and stereochemistry are all determined by the

unique structures of each FAS/PKS complex and its component enzymes.

nascent polyketide undergoes a single cyclisation catalysed by the minimal PKS and is diverted via a shunt pathway by standard chemical means. These experiments established that the KS, CLF and ACP constitute a minimal PKS sufficient for polyketide biosynthesis *in vivo*. The activity of whole [12,13] and minimal PKSs *in vitro* [13] was also recently demonstrated in cell-free enzyme preparations, supporting the conclusions drawn from the experiments *in vivo*.

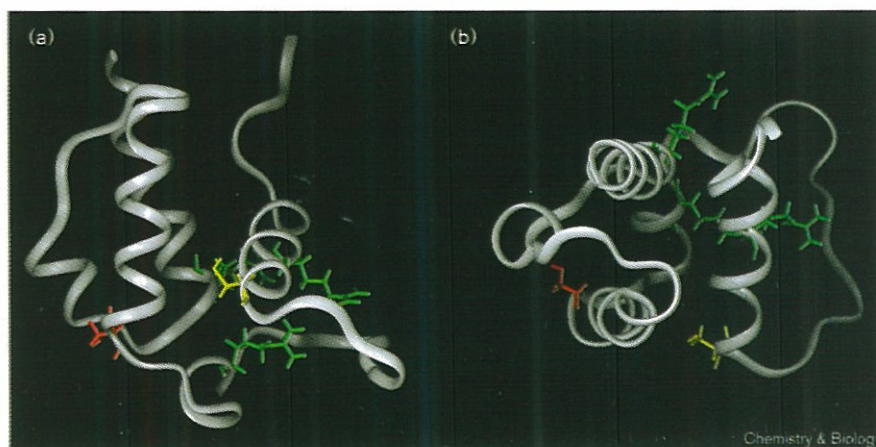
The acyl carrier protein

The best characterised component of type II PKSs is the ACP, which was shown to be essential for polyketide assembly [14], although its precise role in the biosynthetic

process has not been fully elucidated. The protein is expressed in the inactive apo form and is post-translationally modified to the active holo-ACP by transfer of 4'-phosphopantetheine from CoA to a strictly conserved serine residue, catalysed by the enzyme holo-ACP synthase (ACPS). By co-expressing ACP genes with the *acpS* gene from *E. coli*, large quantities of several type II PKS holo-ACPs were recently made available for the first time [15]. The solution structure of the actinorhodin (act) ACP was solved using two-dimensional nuclear magnetic resonance (NMR) methods and was shown to have the same four helix motif as the *E. coli* FAS ACP (Figure 3) [16,17]. In contrast to the *E. coli* ACP, however, the corresponding hydrophobic cleft of the act ACP contains several

Figure 3

The solution structure of actinorhodin (act) PKS ACP [16]. The structure is represented by ribbon diagrams with several significant residues highlighted. (a) Helix 1 is on the right separated from helices 2 (front), 3 (left) and 4 (back) by the putative binding cleft. (b) A 90° forward rotation of (a) looking at the structure down helices 2 and 4. Ser42 (red) is the strictly conserved site of phosphopantetheinylation; Cys17 (yellow) forms a disulphide bond with the pantetheine thiol of wild-type act holo-ACP. A mutant act C17S holo-ACP has been engineered to overcome this problem (J.C., K.J.B., T.S.H., R.J.C., M.P. Crump, I.S.C. Findlow, M.J. Bibb and T.J.S., unpublished observations). Arg11, Arg34 and Arg72 (green) are well conserved amongst type II PKS ACPs and their sidechains protrude into the putative binding cleft of the ACP.



hydrophilic residues, consistent with the ACP being involved in the stabilisation of a highly functionalised carbon chain. In order to study interactions between polyketide assembly intermediates and holo-ACPs and between acylated ACPs and other components of type II PKSs, facile methods of acylation are required. We have recently characterised the rapid chemical acylation of the act ACP by reaction with acyl imidazolides (J.C., K.J.B., T.S.H., R.J.C., M.P. Crump, I.S.C. Findlow, M.J. Bibb and T.J.S., unpublished observations).

Acyl transferase enzymes

Malonylation of FAS ACPs *in vivo* is catalysed by malonyl transferase (MT) enzymes, which promote the transfer of extender units from malonyl CoA to the pantetheine thiol of the holo-ACP. All MT enzymes from fatty acid and polyketide biosynthesis identified so far have in common a highly conserved active-site motif centred around a nucleophilic serine residue [18]. The malonyl extender unit is believed to be transiently bound as an ester to the serine's hydroxyl group [19] and, as a result, the malonyl transferase activity of the *E. coli* MT enzyme can be inhibited using the serine protease inhibitor phenylmethylsulphonyl fluoride (PMSF) [20]. Varying specificities are exhibited by the known malonyl transferases [21–24]; the archetypal type II FAS from *E. coli* has a discrete MT subunit that is specific for malonate [19]. In contrast, no genes encoding discrete MT enzymes have been identified in any of the type II PKS gene clusters characterised so far [4,8]. Proteins with malonyl-CoA:ACP transacylase activity have been isolated from *S. coelicolor* [25] and *Streptomyces glaucescens* [26] and the corresponding genes have been cloned. The MT gene (*fabD*) in *S. coelicolor* was mapped to a putative FAS gene cluster and it was proposed that this enzyme catalyses malonylation of both FAS and PKS ACPs [25].

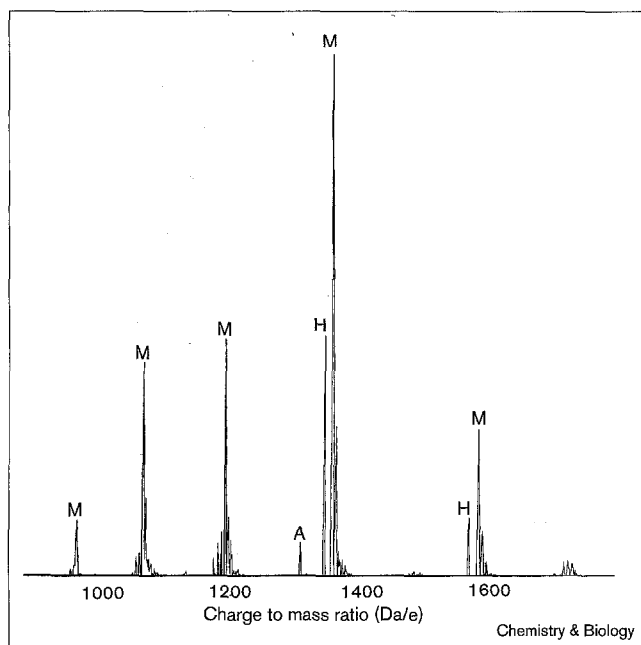
In this paper we present evidence that type II PKS ACPs are capable of self-catalysing the transfer of malonate and a variety of other acyl groups from the corresponding acyl-CoA derivatives to the pantetheine thiol. The reaction has been chemically and kinetically characterised and its use for the synthesis of acyl-ACP derivatives in sufficient quantities for structural studies has been investigated.

Results

Self-malonylation by act C17S holo-ACP

In the course of experiments to determine the substrate specificity of recombinant malonyl transferase from *S. coelicolor* ([25]; T.S.H., R.J.C., J.C. and T.J.S., unpublished observations) act Cys17→Ser (C17S) holo-ACP was incubated with malonyl CoA in phosphate buffer at 30°C. To our surprise, a monomalonyl adduct formed rapidly and malonyl addition was observed by electrospray mass spectrometry (ESMS) to be complete after 30 min (Figure 4). (The C17S mutant act ACP was used in these initial experiments to overcome complications with the wild-type

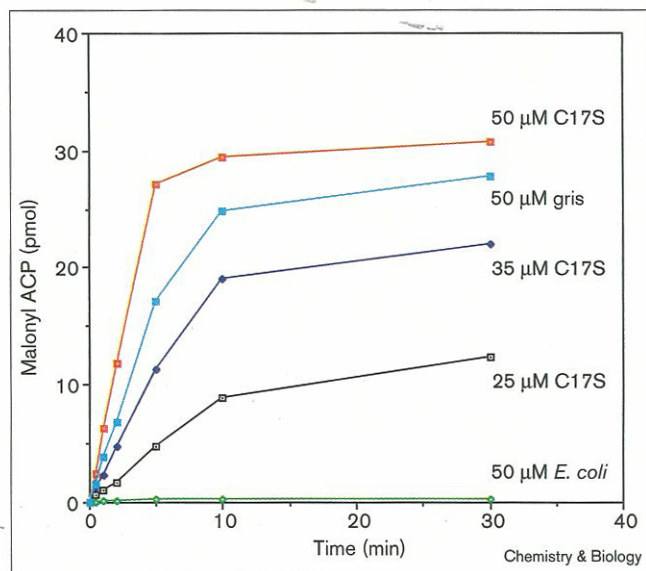
Figure 4



An electrospray mass spectrum showing the extent of malonylation of act C17S holo-ACP after 20 min. A trace amount of apo-ACP in the sample illustrates that malonylation of the apo protein did not occur. Act C17S apo-ACP (A): calculated mass = 9101 Da, observed mass = 9,101.80 Da; act C17S holo-ACP (H): calculated mass = 9,441 Da, observed mass = 9,441.63 Da; malonyl adduct (M): calculated mass = 9,527 Da, observed mass = 9,527.71 Da.

protein, caused by the formation of an intramolecular disulphide bond between the pantetheine thiol and Cys17 (J.C., K.J.B., T.S.H., R.J.C., M.P. Crump, I.S.C. Findlow, M.J. Bibb and T.J.S., unpublished observations) but the wild type protein behaves similarly.) No malonylation of act C17S apo-ACP was observed under identical conditions to the act C17S holo-ACP experiment and a similar incubation of act holo-ACP with acetyl CoA failed to generate the acetyl adduct, even after prolonged incubation (8 h). To confirm that malonylation of the holo protein had occurred on the pantetheine chain, the thiol group was blocked by the addition of the thiol specific reagent *p*-nitrophenyldisulphide. Subsequent incubation of the derivatised holo-ACP with malonyl CoA did not result in any malonyl adduct formation, as expected. Initial analysis of the rate of the malonylation reaction using ¹⁴C-labelled malonyl CoA gave a reaction profile showing initial fast malonylation to an apparent equilibrium position (Figure 5). The results of these experiments are all consistent with the holo-ACP being capable of self-catalysing malonylation of its pantetheine prosthetic group.

Self-malonylation has not been observed previously in studies on either FAS or PKS ACPs and we did not observe any addition of either malonate or acetate to the *S. coelicolor* FAS ACP [27] after 8 h. The recombinant act

Figure 5

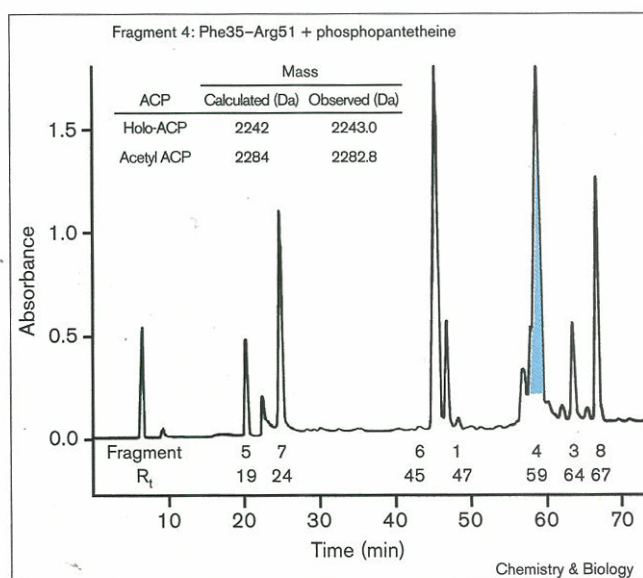
The malonylation of various ACPs as a function of time. Incubations were performed as described in the Materials and methods section, except that the ACP concentration was varied where indicated. The malonyl CoA concentration was 50 μ M.

C17S holo-ACP used in our initial studies was purified from *E. coli* pRJC001 [15] and the purity of the protein had been determined as greater than 99% by both sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) and ESMS (data not shown). It was still possible that trace quantities of the *E. coli* FAS malonyl transferase (MT) were present in the ACP sample, however. Although this was considered unlikely, because the difference in physical properties of the two proteins makes co-purification of significant quantities of MT with the PKS ACP improbable [20,28], the observed specificity of the reaction for malonate and not acetate matched the specificity of *E. coli* MT. As mentioned above, Joshi and Wakil [20] reported that PMSF is an effective inhibitor of *E. coli* MT activity (91% inhibition of 1.8 μ M *E. coli* MT by 1 mM PMSF). Repeating the malonylation reaction with act C17S holo-ACP in the presence of 2 mM PMSF caused no decrease in the rate. To ensure that there was no interaction between PMSF and the ACP the reaction was also monitored by ESMS. The formation of PMSF adducts was not observed either before or after complete malonylation of the protein, and the extent of derivatisation was unaffected. Based on the apparent purity of the ACP sample, any MT present would be at a concentration of less than 0.5 μ M and substantial inhibition of this MT by PMSF would be expected.

The ACP sequesters malonate from bulk solvent

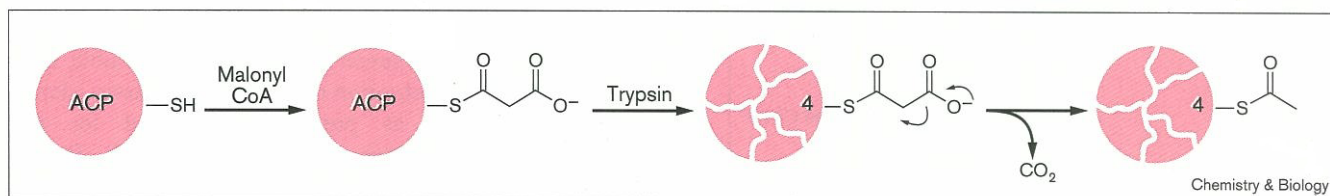
To further confirm that the site of malonylation of the act C17S holo-ACP is the pantetheine thiol, the protein was

malonylated, subjected to trypsin proteolysis and the peptide fragments were separated using high performance liquid chromatography (HPLC) on a C_{18} reverse-phase column. As expected, seven of the eight observable fragments had identical retention times (R_t) and masses (determined by ESMS) as previously observed for the underivatised holo protein (J.C., K.J.B., T.S.H., R.J.C., M.P. Crump, I.S.C. Findlow, M.J. Bibb and T.J.S., unpublished observations). The exception was the fragment containing pantetheine, which corresponds to residues 35–51. Although the retention time was identical to that previously observed, the mass of the fragment was 42 Da higher, equivalent to an acetyl adduct rather than the expected malonyl adduct (Figure 6). We believed that the observed acetylated fragment resulted from decarboxylation of the malonylated peptide under either the proteolysis incubation or ESMS conditions (Figure 7). Several experiments were conducted to investigate this decarboxylation reaction. Malonyl CoA was incubated in turn with 100 mM NH_4HCO_3 at pH 8, 0.1% TFA and 1% formic acid to simulate the proteolysis incubation, HPLC and ESMS conditions, respectively. Analysis by ESMS showed that no decarboxylation to form acetyl CoA had occurred. Further incubations of malonyl CoA in the presence of trypsin or proteolytic fragments of the act holo-ACP also

Figure 6

The purification of proteolytic fragments of malonyl act C17S ACP by HPLC. Fragments 1–3 and 5–8 were characterised previously (J.C., K.J.B., T.S.H., R.J.C., M.P. Crump, I.S.C. Findlow, M.J. Bibb and T.J.S., unpublished observations) and their retention times and observed masses remained unaltered upon malonylation. Fragment 4 (Phe35–Arg51) bearing the acetyl pantetheine adduct is highlighted in blue. Insert: ESMS data of fragment 4 demonstrating an acetyl adduct and comparison with the corresponding fragment from underivatised act C17S holo-ACP.

Figure 7



A schematic showing the decarboxylation of the malonyl adduct of fragment 4 from tryptic proteolysis of act malonyl C17S ACP.

failed to cause decarboxylation of the malonyl moiety. It seemed that the decarboxylation of malonyl ACP therefore might be catalysed by some characteristic of the whole or partially digested protein. Malonyl ACP was prepared as described above and incubated under identical conditions to the proteolysis experiments, except that trypsin was omitted. The protein was repurified by HPLC as described previously [28] and analysis by ESMS showed that no decomposition of malonate to acetate had occurred. Incubation of malonylated ACP in 8 M urea also failed to cause decarboxylation of the ACP-bound malonate. This suggests that the malonate is sequestered by the holo-ACP and that the decarboxylation of the ACP-bound malonate could be catalysed by a residue of the holo-ACP that only becomes accessible after partial proteolytic cleavage. To examine whether self-malonylation requires the native structure of the protein, act C17S holo-ACP was denatured in 8 M urea at 30°C for 4 h prior to the addition of malonyl CoA. After incubation for 1 h no malonylation of the holo-ACP was observed. To investigate further the effect of the ACP structure on the malonylation reaction, holo-ACP was incubated in urea at a range of concentrations between 0.5 and 8 M for 4 h, then in the presence of malonyl CoA for 1 h. Concentrations of 0.5–2 M urea did not affect the extent of malonylation, but at higher urea concentrations the malonylation reaction was inhibited to a successively greater extent until malonylation was fully inhibited in 6 M urea. These results all confirm that the ACP must retain at least part of its native structure to catalyse the self-acylation reaction. Interestingly, at 0.5 M urea a small peak corresponding to acetyl ACP (5% of acyl ACP; calculated molecular weight (M_r) = 9483; observed M_r = 9483.75), presumably from decarboxylation of the malonyl adduct, was observed. The extent of decarboxylation increased with increasing urea concentration until at 4 M urea the acetyl adduct predominated (90% of the ACP was acetylated rather than malonylated). These experiments provide firm evidence that, under certain conditions, that is partial denaturation of the ACP either as a result of urea denaturation or trypsin degradation, decarboxylation of a malonyl moiety covalently bound to the phosphopantetheine group can be observed. This decarboxylation does not occur when malonyl ACP is fully denatured, nor when it retains its fully native structure.

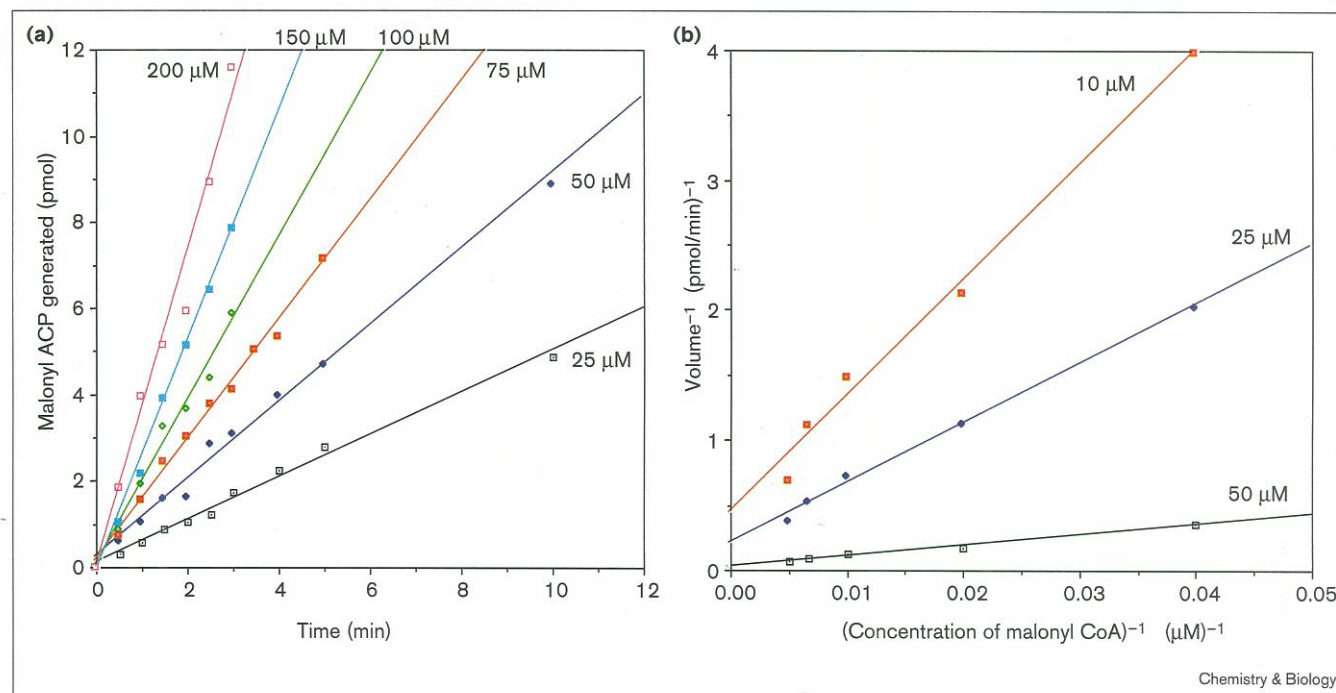
Kinetic characterisation of the malonylation reaction

As expected, the rate of self-malonylation of act C17S holo-ACP varies with ACP concentration (Figure 5). Reaction under the same conditions of the griseus B (gris) holo-ACP (Figure 5) and oxytetracycline (otc) holo-ACP (data not shown) proceeded at slightly slower rates, but no malonylation of *E. coli* ACP was observed. To investigate the kinetics of the reaction, the rate of malonylation was measured as a function of malonyl CoA concentration at several fixed concentrations of act C17S holo-ACP (Figure 8). The data were plotted on reciprocal coordinates to give a series of divergent lines, typical of Michaelis–Menten kinetics. From this plot a K_m value of 219 μM was estimated for malonyl CoA and a catalytic constant of the reaction (k_{cat}) was calculated to be 0.34 min^{-1} , and the specificity constant k_{cat}/K_m was $1.55 \times 10^{-4} \mu\text{M}^{-1}\text{min}^{-1}$. This reaction is without precedent and so comparison with the complex kinetics of malonyl transferase reactions [20] may be invalid. To assess the relevance of the kinetic constants we investigated the reversibility of this self-malonylation reaction. Act C17S holo-ACP was incubated with a ten molar excess of malonyl CoA until malonylation was complete, then a ten molar excess of free CoA was added and the effect of this on the extent of malonylation was monitored by ESMS. The degree of malonylation decreased, and after 4 h more than 75% of the ACP had been de-malonylated. This reversibility suggests that this reaction is a truly catalytic process.

Mechanistic characterisation of self-malonylation

Rangan and Smith [22] recently reported that mutation of a single arginine residue (Arg606) in the AT/MT domain of the rat FAS results in a 99% loss of MT activity, and a sixfold increase in AT activity. They proposed that the guanidino group of Arg606 forms a salt bridge with the malonyl carboxylate, anchoring the malonyl moiety in the active-site pocket. We have previously noted [16] that the putative binding cleft of the act holo-ACP contains several arginine residues that are well conserved amongst type II PKS ACPs but are poorly conserved amongst FAS ACPs. The specificity of the self-acylation reactions of PKS ACPs for malonate but not acetate suggests that a binding mechanism similar to that proposed for the FAS MT might be invoked. To probe the mechanism of the

Figure 8



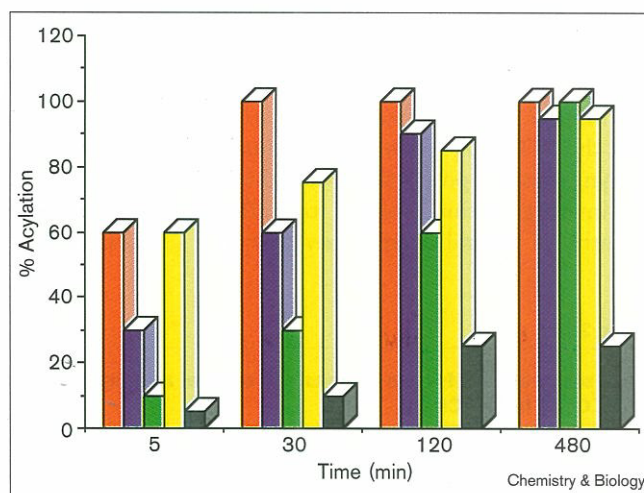
The kinetic analysis of the self-malonylation of act C17S holo-ACP. All assays were performed as described in the Materials and methods section. (a) Rate curves for the reaction as a function of malonyl CoA concentration at fixed ACP concentration (25 μM).

(b) Lineweaver-Burk data for the self-malonylation reaction at several ACP concentrations. Kinetic constants calculated from the plot were $K_m = 219 \mu\text{M}$, $k_{\text{cat}} = 0.34 \text{ min}^{-1}$, $k_{\text{cat}}/K_m = 1.55 \times 10^{-4} \mu\text{M}^{-1} \text{ min}^{-1}$.

malonylation reaction we investigated the synthesis of a range of acyl derivatives of several PKS ACPs (Figure 9). Malonylation of the wild-type act, gris and otc ACPs as well as the act C17S ACP was rapid and complete (100% after 30–60 min; Table 1), although the rates of reaction of the gris and otc ACPs were slower than the rates observed for the act proteins. Complete addition of both methylmalonyl and acetoacetyl to the PKS ACPs was also observed (Table 2). Incubation with a ten molar excess of succinyl CoA resulted in only 25% conversion to succinyl ACP, although this level was rapidly reached. If this is a consequence of the position of equilibrium it may be possible to achieve higher conversion levels using higher concentrations of substrate. No acylation was observed on incubation of the protein with butyryl CoA. These observations are consistent with formation of a salt bridge in which the position of the carboxylate moiety on the acyl chain is important. We reasoned that acetoacetate, the first condensation product of both FAS and PKS systems, would also be capable of salt bridge formation via its enolate tautomer (Figure 10) and we have previously proposed this as a mechanism for polyketide chain stabilisation [16], although simple hydrogen bonding to the β -keto ester is also possible. As predicted, an acetoacetyl adduct was formed upon incubation of the ACPs with acetoacetyl CoA and this acylation was complete after 8 h.

FAS [29,30] and PKS ACPs can be chemically acylated using acyl imidazolide species, but it has been reported

Figure 9



The synthesis of acyl ACP derivatives. Acylation of act C17S holo-ACP using a variety of acyl CoAs and acetoacetyl NAC. Malonyl CoA (red), methylmalonyl CoA (blue), acetoacetyl CoA (green), acetoacetyl NAC (yellow), succinyl CoA (black). The extent of acylation was estimated from the relative peak areas in the transformed electrospray mass spectra.

Table 1

Mass spectral data of ACPs and their malonyl derivatives.

Holo-ACP	Calculated mass (Da)	Observed mass (Da)
Act C17S	9,441	9,441.49
+ acetyl	9,483	—
+ malonyl	9,527	9,527.71
Act wild type	9,457	9,456.91
+ acetyl	9,499	—
+ malonyl	9,543	9,546.45
<i>S. coelicolor</i> FAS	9,124	9,125.97
+ acetyl	9,166	—
+ malonyl	9,210	—
Gris (– methionine)	9,884	9,880.5
+ acetyl	9,926	—
+ malonyl	9,970	9,979.52
Gris (+ methionine)	10,016	10,015.24
+ acetyl	10,058	—
+ malonyl	10,102	10,101.28
Otc	10,256	10,250.38
+ acetyl	10,298	—
+ malonyl	10,342	10,340.90

Act, actinorhodin; gris; griseusin; otc; oxytetracycline.

that *S*-acyl derivatives of *N*-acetyl cysteamine (NAC) are not sufficiently reactive for acylation of the FAS protein to occur [29]. To investigate whether the self-acylation reaction of type II PKS ACPs could be extended to acyl NAC species, acetoacetyl NAC was incubated with act C17S holo-ACP. Extensive acylation (95%) at a rate comparable to the reaction of acetoacetyl CoA was observed (Figure 9), demonstrating that specific interactions between the ACP and the CoA moiety may be insignificant. The success of this reaction may enable a wide range of β -keto acyl ACP derivatives to be synthesised.

Comparison of malonyl transfer and self-malonylation

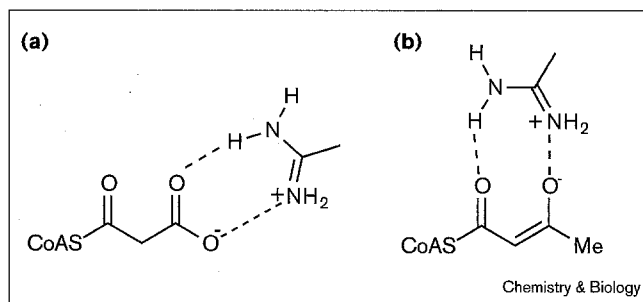
Revill *et al.* [25] and Summers *et al.* [26] both proposed that the MT enzyme might be a link between fatty acid biosynthesis and polyketide biosynthesis in streptomycetes. To investigate this proposal the effect of an equimolar quantity of recombinant *S. coelicolor* MT (T.S.H., R.J.C.,

Table 2

Mass spectral data for the synthesis of several acyl derivatives of act C17S holo-ACP.

	Conversion (%)	Calculated (Da)	Observed (Da)
C17S ACP	—	9,441	9,441.63
Malonyl	100	9,527	9,527.71
Methylmalonyl	95	9,541	9,542.25
Succinyl	25	9,541	9,543.75
Acetoacetyl (CoA)	100	9,525	9,525.75
Acetoacetyl (NAC)	95	9,525	9,527.09

Figure 10



The formation of salt bridges by the guanidino sidechain of arginine residues. (a) The interaction with the carboxyl group of malonate. (b) The interaction with the enolate tautomer of acetoacetate.

J.C. and T.J.S., unpublished observations) on the self-malonylation of act C17S holo-ACP was measured (Figure 11). The reaction between the MT protein and malonyl CoA to form a malonyl adduct rapidly reached equilibrium and the rate of reaction was too fast to be measured, consistent with reported studies on the *E. coli* MT [19]. The competition of the MT enzyme for the substrate was found to slow the initial rate of act ACP self-acylation. After longer incubation times, however, the rate of reaction approached that of the self-malonylation in the absence of MT. As no acceleration of the ACP malonylation rate was observed we suggest that the act ACP is a poor substrate for the *S. coelicolor* FAS MT and that the FAS and PKS complexes may therefore function independently *in vivo*.

Discussion

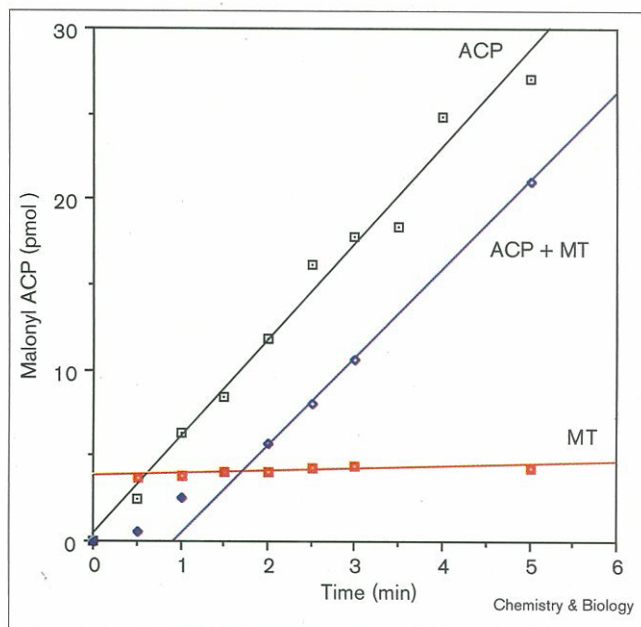
The apparent roles of many of the type II PKS subunits *in vivo* were elucidated through combinatorial manipulation of the PKS genes [10]. This established that the minimal set of proteins for polyketide synthesis *in vivo* is comprised of the KS, CLF and ACP [31]. This differs from the *E. coli* FAS for which there are at least two possible mechanisms for the initial steps of fatty acid synthesis. The combinations of KSIII with acetyl CoA and malonyl ACP or KSI (or KSII) with acetyl ACP and malonyl ACP are both sufficient for initial chain elongation [32]. Active cell-free PKS complexes have also been obtained [12,13], but the only component of type II PKSs that has been purified and characterised to any extent *in vitro* is the ACP ([28]; J.C., K.J.B., T.S.H., R.J.C., M.P. Crump, I.S.C. Findlow, M.J. Bibb and T.J.S., unpublished observations). We had previously developed a technique for the heterologous expression and isolation of large quantities of type II PKS ACPs, predominantly in the active holo form [15]. Characterisation of the act holo-ACP revealed several differences between the PKS protein and its FAS counterparts. Although the primary sequences and global folds of the PKS and FAS ACPs are strikingly similar, the act protein has been observed in

only one conformation in solution [16], compared with the two or more distinct conformations proposed for the *E. coli* protein [33]. In addition, the different polarities of the ACP clefts were proposed to be significant in terms of the binding of assembly intermediates. We observed a biochemical incompatibility of PKS ACPs with an *E. coli* FAS subunit, the acyl ACP synthetase (J.C., K.J.B., T.S.H., R.J.C., M.P. Crump, I.S.C. Findlow, M.J. Bibb and T.J.S., unpublished observations). This correlates well with the reported low ability of FAS ACPs from *S. coelicolor* [11,25] and *Saccharopolyspora erythraea* [34] to replace the act PKS ACP *in vivo* and of FAS ACPs from *S. glaucescens* and *E. coli* to fully complement a cell-free tetracenomycin PKS lacking ACP [12]. Here we describe the first observation of catalytic self-malonylation by type II PKS holo-ACPs. This represents another departure from the analogy between FAS and PKS systems and we propose that it may provide a key to understanding the role of the ACP in polyketide biosynthesis.

Mechanism of self-malonylation

The solution structures of the act (Figure 3) [16], gris and otc (M.P. Crump, I.S.C. Findlow, C.E. Dempsey, J.A. Parkinson, J.C., T.J.S., unpublished observations) ACPs were recently solved using two-dimensional and three-dimensional NMR methods. With these structures in hand, a primary target of further work is to derivatise PKS holo-ACPs with polyketide assembly intermediates in order to investigate the role of the ACP in the stabilisation of these highly functionalised carbon chains. Although chemical acylation of both FAS [29,30] and PKS ACPs (J.C., K.J.B., T.S.H., R.J.C., M.P. Crump, I.S.C. Findlow, M.J. Bibb, and T.J.S., unpublished observations) using acyl imidazolide species is possible, the difficulty in synthesising the highly reactive acyl imidazolides of the more labile polyketide assembly intermediates is limiting. Enzymatic synthesis of acyl PKS ACPs using *E. coli* acyl ACP synthetase is prevented by the biochemical incompatibility of the FAS subunit and the PKS ACPs (J.C., K.J.B., T.S.H., R.J.C., M.P. Crump, I.S.C. Findlow, M.J. Bibb, and T.J.S., unpublished observations). We have previously reported the enzymatic synthesis of acetyl act ACP using holo-ACPS from *E. coli* and act apo-ACPs with acetyl CoA [15]. The general use of this reaction in enzymatic synthesis has yet to be determined, but it may be low because the PKS ACPs are poorer substrates for the ACPS than the *E. coli* FAS ACP [35]. In contrast, the malonyl transfer from malonyl CoA to the pantetheine thiol of the ACP described here is rapid and facile, and has the added advantage that the malonyl ACP can be purified easily once synthesis is complete. It is therefore of considerable interest to determine the mechanism and use of the reaction for the synthesis of acyl ACP derivatives. We were initially concerned that the malonyl transfer was catalysed by trace impurities of the *E. coli* FAS MT, but the failure of PMSF to inhibit the reaction renders this unlikely.

Figure 11



The effect of *S. coelicolor* malonyl transferase (MT) on the self-malonylation of act C17S ACP ([25]; T.S.H., R.J.C., J.C. and T.J.S., unpublished observations). Incubation of MT with malonyl CoA alone rapidly reaches equilibrium. Co-incubation of MT with ACP does not result in acceleration of the rate of malonylation of the ACP, but instead causes a slowing of the initial rate of reaction because of competition for the malonyl CoA substrate.

Acylation of type II PKS ACPs upon incubation with malonyl, methylmalonyl and succinyl CoAs, but not with acetyl or butyryl CoAs suggests that the carboxylate functionality of the malonyl analogues is mechanistically important. Proteolysis of act malonyl C17S ACP under basic conditions resulted in decarboxylation of the malonate bound to the ACP to form an acetyl peptide adduct. Incubation of holo-ACP with malonyl CoA at urea concentrations of 0–8 M suggested that this decarboxylation was catalysed by one or more residues in the protein that only become accessible upon partial denaturation of the ACP. In addition, Rangan and Smith [22] reported that an active site arginine of the animal FAS MT domain is involved in malonyl transfer, but not acetyl transfer, by binding to the malonyl carboxylate via a salt bridge. We proposed previously that conserved arginine residues within the hydrophobic cleft of the ACP might be involved in the stabilisation of poly- β -keto assembly intermediates [16]. We now propose, therefore, that the malonyl moiety is sequestered by the ACP, and it seems likely that one of the ACP arginines may play a similar anchoring role in the self-malonylation reaction. These arginines are well conserved amongst type II PKS ACPs, but are poorly conserved amongst FAS ACPs [16], which is consistent with the observed inability of FAS ACPs to catalyse self-malonylation. The slower rate of acylation using methylmalonate

could then be a result of increased steric hindrance caused by the pendant methyl moiety. Interestingly, the much lower rate and extent of reaction observed with succinyl CoA suggests that formation of a salt bridge is dependent on the position of the carboxylate group relative to the acyl thioester linkage. The complete acylation of the PKS ACPs using acetoacetyl CoA provided further insight into the mechanism of polyketide assembly. Although acetoacetate lacks a carboxylate functionality, binding within the ACP cleft may occur by simple hydrogen bonding to the β -keto ester or via a salt bridge with the enolate tautomer (Figure 10). Thus it seems that the ACP can bind both malonate extender units and polyketide assembly intermediates in the cleft using one or more of the conserved arginine residues, stabilising the acyl species in the process. We are currently investigating the catalytic role of these conserved arginines by site-directed mutagenesis and further kinetic and mechanistic studies.

Scope for the synthesis and study of acyl ACPs

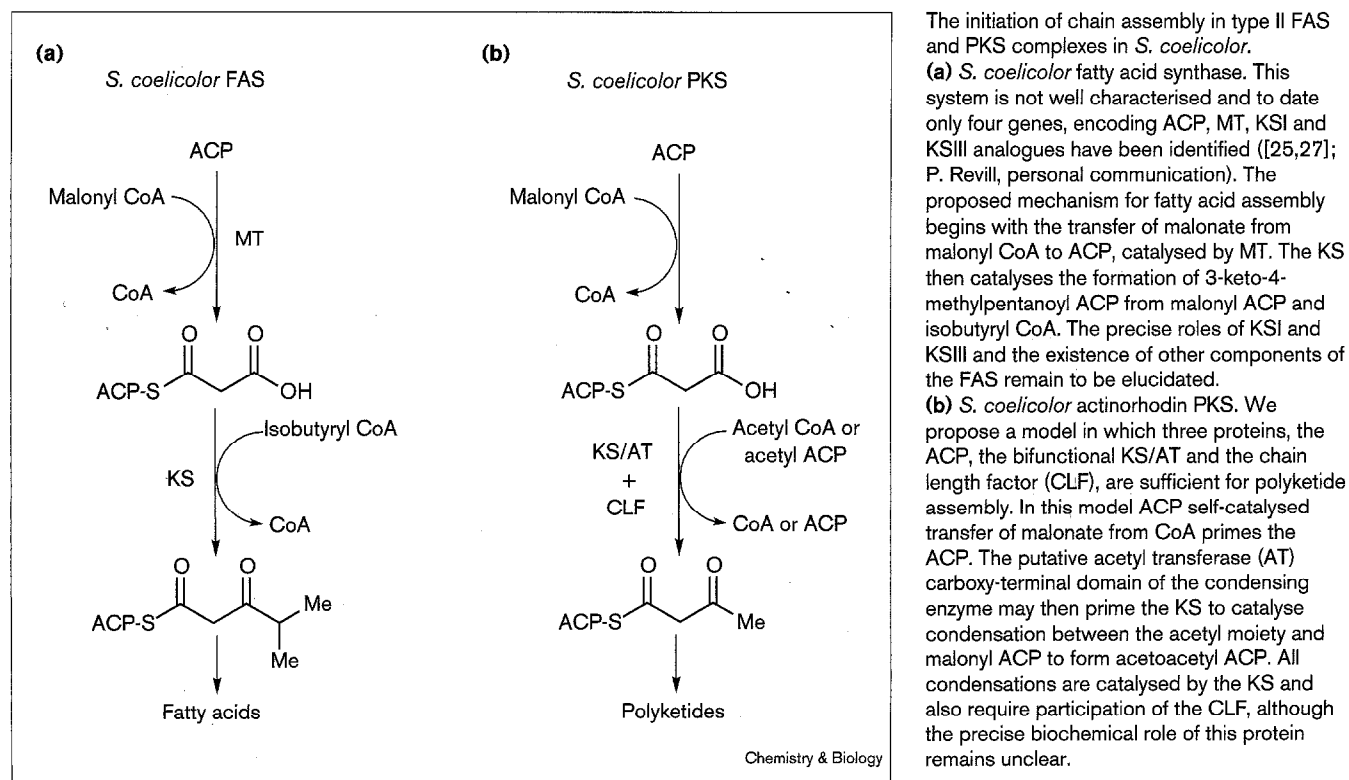
During chemical acylation studies on the *S. erythraea* FAS ACP Bridges *et al.* [29] incubated this ACP with *S*-acyl derivatives of NAC and did not observe any acylation, even after 24 h. In contrast, nearly complete derivatisation of act C17S ACP occurred upon incubation with acetoacetyl NAC; the rate of this reaction was similar to the reaction with acetoacetyl CoA, effectively ruling out any significant

binding between the ACP and all but the distal portion of the CoA pantetheine chain. This reaction markedly broadens the scope for synthesis of acyl ACPs. Our group [36] and others [37] have prepared many polyketide assembly intermediates as their NAC thioesters in order to probe the biosynthetic pathways leading to polyketide metabolites. The relative ease with which chemical synthesis of acyl derivatives of NAC using polyketide assembly intermediates can be achieved makes using these acyl thioesters for the synthesis of acyl ACPs an enticing prospect. At this stage, it appears that acylation of type II PKS ACPs may be possible using any of these compounds which contain a keto group adjacent to the acyl thioester bond.

A new model for the initiation of polyketide biosynthesis

The rapid malonylation of the recombinant act C17S holo-ACP (Figures 4, 5) suggests a unique mechanism for the initiation of polyketide synthesis by type II PKSs. Discrete acyl transferase domains have not been identified in any of the gene clusters encoding the type II PKSs. On the basis of sequence analysis, it was proposed that the 3'-terminal half of the KS gene encodes an AT domain [38], but site-directed mutagenesis of the putative active-site serine failed to eliminate polyketide production [39,40]. Until the putative bifunctional KS/AT protein is isolated and characterised, however, the role of this domain will remain unclear. Complementary to this

Figure 12



work was the isolation of MT enzymes from both *S. coelicolor* and *S. glaucescens* which are apparently capable of malonylating both FAS and PKS ACPs [25,26]. A model was proposed in which the malonyl transfer in both fatty acid and polyketide pathways in streptomycetes would be catalysed by the FAS MT enzyme. Revill *et al.* [25] used act ACP (2–3% holoform) to identify and follow the purification of the *S. coelicolor* FAS MT. Summers *et al.* [26] similarly used a tetracenomycin (tcm) ACP sample that was predominantly in the inactive apo form [41] in an MT activity assay. The level, if any, of malonylation of the tcm ACP in the absence of the *S. glaucescens* MT was not reported. The recombinant *S. coelicolor* MT is capable of catalysing the malonylation of both *E. coli* ACP (T.S.H., R.J.C., J.C., T.J.S., unpublished observations) and *S. coelicolor* FAS ACP [27] and our results demonstrate that this enzyme binds malonate, as would be expected by analogy to the *E. coli* MT. No increase in the rate of malonylation of act C17S holo-ACP was observed when incubated with an equimolar quantity of the *S. coelicolor* MT and malonyl CoA, however. It is possible that the reported conditions of low holo-ACP, malonyl CoA and MT concentrations (under which malonyl transfer from CoA to act ACP was observed to be stimulated by the MT [25,26]) were suboptimal for detection of ACP self-malonylation. Given that we now report the self-loading of malonate by the type II PKS ACPs (an activity not observed for the type II FAS ACPs under the same conditions), we propose an alternative model for the initiation of aromatic polyketide biosynthesis in streptomycetes (Figure 12). ACP-catalysed self-malonylation, together with the uptake of acetate, derived from acetyl CoA (or acetyl ACP formed by decarboxylation of ACP-bound malonate) by the KS/AT, possibly assisted by the CLF, would initiate chain extension in the absence of any other enzymes. In this model only acetyl CoA and malonyl CoA are required and the KS/CLF/ACP complex would constitute a truly minimal PKS. This model is fully consistent with the observed activity of purified type I PKS enzymes *in vitro* [42] and components of the *E. coli* type II FAS [43], which are apparently capable of acquiring the necessary acyl starter by decarboxylating the ACP-bound extender unit. Thus, although there is wide variety both in the arrangement of AT and MT activities and in the mechanism of specificity, the ability of each type of FAS or PKS to catalyse chain assembly from acyl CoAs *in vitro* could be self-contained. The reported ability of *Streptomyces* FAS MT enzymes to catalyse the malonylation of PKS holo-ACPs under certain conditions, however, suggests that there may be more than one mechanism by which aromatic polyketide biosynthesis is initiated *in vivo*. Synthesis of acyl ACPs via the self-acylation reaction will enable a wide range of chemical, biochemical and structural studies to be carried out using purified individual type II PKS subunits and reconstituted synthase complexes *in vitro*.

Significance

The acyl carrier protein (ACP) is a vital component of type II polyketide synthase (PKS) multienzyme complexes. Its specific role in the polyketide biosynthetic pathway, other than as a carrier of the nascent polyketide chain, has remained enigmatic, however. The recent development of a method to generate large quantities of type II PKS ACPs in the active holo form has made the *in vitro* study of these proteins possible. We have observed that the holo-ACP can self-catalyse the transfer of malonate from malonyl CoA to its pantetheine thiol. Using the serine-specific inhibitor phenylmethylsulphonyl fluoride (PMSF), we have demonstrated that this malonyl transfer is not a result of contaminating quantities of the *Escherichia coli* malonyl transferase (MT) protein. This activity marks a novel distinction between the type II FAS and PKS complexes.

We have proposed a new model for the initiation of polyketide assembly in type II PKSs—in which the holo-ACP catalyses its own malonylation. Complementary acetyl transfer to the β -ketoacyl synthase (KS) active-site thiol catalysed by the carboxy-terminal domain of the putative β -ketoacyl synthase/acyl transferase (KS/AT) bifunctional protein would facilitate chain elongation. A truly minimal PKS would therefore comprise only the ACP, KS/AT and chain lengthening factor (CLF) proteins. Kinetic evidence suggests that self-malonylation by the ACP proceeds by a similar mechanism to the MT-catalysed reaction in other FAS and PKS systems. We have proposed that an arginine in the putative binding cleft of the ACP may bind the malonyl moiety via a salt bridge.

The relaxed substrate specificity exhibited by the PKS ACPs provides a facile route for the synthesis of acyl ACP derivatives using a wide range of polyketide assembly intermediates. Structural studies using two-dimensional and three-dimensional NMR methods on these acyl ACPs should provide further insight into the role of the ACP in polyketide biosynthesis.

Materials and methods

Expression, purification, acylation and ESMS analysis of PKS ACPs

The expression and purification of PKS holo-ACPs was as described previously [14,28]. To avoid complications caused by the formation of an internal disulphide bond by act wild-type holo-ACP, the act C17S ACP (J.C., K.J.B., T.S.H., R.J.C., M.P. Crump, I.S.C. Findlow, M.J. Bibb, and T.J.S., unpublished observations) was used, unless otherwise stated. Typically the assay contained 50 μ M ACP, 0.5 mM acyl CoA, 1 mM dithiothreitol (DTT) and 50 mM potassium phosphate (pH 7.5) in a final volume of 200 μ L. The reaction was initiated by the addition of the acyl CoA (20 μ L) to the ACP, which had been equilibrated for 10 min at 30°C. Aliquots (20 μ L) were removed at timed intervals up to 8 h and quenched by the addition of 5 μ L 25% formic acid. Analyses were performed immediately. Mass spectra were measured as previously described (J.C., K.J.B., T.S.H., R.J.C., M.P. Crump, I.S.C. Findlow, M.J. Bibb, and T.J.S., unpublished observations) on a VG

Quattro spectrometer equipped with an electrospray ion source. Derivatisation of the act holo-ACP with *p*-nitrophenyldisulphide was as previously described (J.C., K.J.B., T.S.H., R.J.C., M.P. Crump, I.S.C. Findlow, M.J. Bibb, and T.J.S., unpublished observations). The reaction of acetoacetyl NAC was performed as described above except that the NAC thioester was dissolved in CHCl_3 so that the organic solvent comprised less than 2% (v/v) of the reaction mixture.

Reversibility of malonylation

Malonyl ACP (50 μM) was synthesised as described above in a final volume of 400 μl . Free CoA was then added to 0.5 mM and the reaction was incubated at 30°C removing 20 μl aliquots at timed intervals for analysis by ESMS.

Kinetic analysis of enzymatic acylation of ACPs

All acylation experiments were carried out at 30°C. Typically, the assay mixtures contained 50 mM potassium phosphate (pH 7.5), 50 μM ACP, 41 μM cold malonyl CoA, 9 μM [^{14}C]-malonyl CoA (50 mCi/mmol; New England Nuclear) and 1 mM DTT in a final volume of 100 μl . The assay was started by the addition of 10 μl malonyl CoA to the ACP which had been equilibrated at 30°C for 10 min. Aliquots (10 μl) were removed at timed intervals and reactions quenched by addition to 30 μl ice cold 50% TCA followed by addition of 10 μl BSA (10 mg/ml) as a carrier. Protein was left to precipitate on ice for 15 min and then pelleted by microcentrifugation at 13,000 rpm for 5 min in a Sorvall MSE microfuge. The supernatant was removed and the pellet was washed twice with 50 μl 50% TCA. The pellet was resuspended in 50 μl 2 M Tris base and 50 μl 2 M NaOH by vigorous mixing followed by incubation at 37°C for 30 min. After vigorous mixing and centrifugation (10 s pulse, 13,000 rpm), the protein solution was added to 10 ml liquid scintillant and counted for radioactivity. All assays were repeated in triplicate and appropriate controls were carried out.

PMSF inhibition of *E. coli* malonyl transferase

The reaction mixture was as described above except that the ACP was incubated with 2 mM PMSF for 30 min at 30°C either prior to or after the malonylation of the protein.

Effect of malonyl CoA:ACP transacylase from *S. coelicolor*

The reaction mixture was as described above except that the ACP was equilibrated with recombinant his₆-malonyl CoA:ACP transacylase (T.S.H., R.J.C., J.C., T.J.S., unpublished observations) for 10 min at 30°C prior to the addition of malonyl CoA. A control incubation which lacked only the ACP was also carried out.

Tryptic proteolysis of ACPs and HPLC of peptide fragments

Malonyl act C17S ACP (100 μM) was digested with 2% (w/w) trypsin (sequencing grade, Sigma) in 100 mM NH_4HCO_3 (pH 8.0) for 4 h at 37°C. The digested protein was immediately injected onto a C₁₈-reverse-phase HPLC column (0.4 × 240 mm, Rainin) equilibrated in water. The peptide fragments were eluted with a 0–70% acetonitrile gradient at 0.5 ml/min, monitoring absorption at 214 nm. Fractions containing peptide fragments were lyophilised, resuspended in water containing 1% formic acid and analysed by ESMS. ACPs and their acyl derivatives were incubated under the same conditions without the trypsin, then repurified by HPLC, lyophilised and analysed by ESMS as described previously [28]. All solvents used for HPLC contained 0.1% TFA. All proteolysis experiments were performed at least twice.

Acknowledgements

We thank David Hopwood and Peter Revill for the gift of *S. coelicolor* FAS holo-ACP and their useful comments on the manuscript, Mark Ginty for synthesising acetoacetyl NAC, Stuart Findlow for supplying otc holo-ACP and Chris Dempsey and Richard Sessions for help with the diagram of act ACP. We also acknowledge one of the referees for suggesting the urea denaturation experiments. This work was supported by grants from the Engineering and Physical Sciences Research Council (EPSRC) and the Biotechnology and Biological Sciences Research Council (BBSRC). Studentships from

the EPSRC (T.S.H.) and School of Chemistry (K.J.B.) are gratefully acknowledged. R.J.C. gratefully acknowledges the receipt of a Royal Society research grant.

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