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The Malonyl Transferase Activity of Type II Polyketide Synthase Acyl Carrier Proteins

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Summary

Acyl carrier proteins (ACPs) play a fundamental role in directing intermediates among the enzyme active sites of fatty acid and polyketide synthases (PKSs). In this paper, we demonstrate that the *Streptomyces coelicolor* (*S. coelicolor*) actinorhodin (act) PKS ACP can catalyze transfer of malonate to type II *S. coelicolor* fatty acid synthase (FAS) and other PKS ACPs in vitro. The reciprocal transfer from *S. coelicolor* FAS ACP to a PKS ACP was not observed. Several mutations in both act ACP and *S. coelicolor* FAS ACP could be classified by their participation in either donation or acceptance of this malonyl group. These mutations indicated that self-malonylation and malonyl transfer could be completely decoupled, implying that they were separate processes and that a FAS ACP could be converted from a non-malonyl-transferring protein to one with malonyl transferase activity.

Introduction

The acyl carrier protein (ACP) is a fundamental component of both fatty acid and polyketide biosynthesis. Its roles include: shuttling acyl intermediates between the enzyme active sites of the fatty acid (FAS) and polyketide (PKS) synthases; stabilization of the nascent fatty acid or polyketide intermediates; and regulation of these pathways [1–3]. In addition, the acylation of ACP provides both starter units (e.g., acetyl, propionyl) and 2-carbon extender units (e.g., malonate) that are required for priming and chain elongation of fatty acids and polyketides. Malonate is loaded onto the phosphopantetheine prosthetic arm of the fatty acid ACP by a malonyl CoA:ACP transacylase (MCAT) [4]. A discrete MCAT has been identified in type II FAS gene clusters of bacteria and plants [5, 6], while type I FASs [7] and modular type I PKSs [8] possess integral acyl transferase (AT)/MCAT domains. For type II PKSs however, there is no discrete MCAT located in the gene cluster. This led a number of groups to propose that the FAS MCAT is coopted into polyketide biosynthesis [4, 9]. In 1998, however, Hitchman et al. demonstrated that PKS ACPs from a variety of type II bacterial PKSs were subject to non-MCAT-catalyzed acylation in the presence of malonyl CoA [10]. This self-catalyzed acylation could also be extended to other dicarboxylic and β-keto acid thiol esters, compounds that are not substrates for MCAT [10, 11].

In addition to self-catalyzed acylation, type II PKS ACPs have been observed to catalyze the transfer of malonate to a recipient type II FAS ACP by Reynolds et al. [12]. Subsequently, however, this observation was retracted, and previous reports of self-malonylation and malonyl transfer were attributed to contamination with the *E. coli* MCAT (FabD) from the *E. coli*-based overexpression systems used [13]. We have, however, recently demonstrated that chemically synthesized *Streptomyces coelicolor* (*S. coelicolor*) actinorhodin (act) PKS ACP self-malonylates in the presence of malonyl CoA. Since such a chemically synthesized ACP cannot be contaminated by MCAT, self-acylation must be an inherent enzymic function of this ACP [14]. After confirming that self-malonylation was an inherent property of polyketide ACPs, we considered it possible that malonyl transfer was also a genuine enzymatic property of these carrier proteins.

We now report the observation of malonyl transfer from type II PKS ACPs to reciprocal PKS or FAS ACPs and show that this activity is distinct from self-malonylation. Malonylated type II *S. coelicolor* FAS ACP is unable to catalyze transfer to other FAS or PKS ACPs. However, we have demonstrated that a single mutation can impart a transferase activity to the previously inactive *S. coelicolor* FAS ACP.

Results

In our previous investigations of acylation reactions with the actinorhodin ACP, we observed that C17 in the wild-type (WT) ACP rapidly forms an intramolecular disulfide with the terminal thiol of the phosphopantetheine prosthetic group, rendering the ACP inactive [15]. To circumvent this, we produced a C17S mutant that showed almost identical activity to the WT protein in all other respects. For this reason, the C17S act ACP has been used in all of our experiments. We first established whether act ACP was capable of transferring malonate to the *S. coelicolor* FAS holo-ACP (*Sc*. FAS holo-ACP). Equimolar concentrations of *Sc.* FAS holo-ACP and malonyl CoA were incubated with increasing concentrations of the C17S *S. coelicolor* actinorhodin holo-ACP (C17S act holo-ACP) (0, 2, 6, and 10 μM) at 30ºC for 30 min, and the reaction mixture was assayed by electrospray mass spectrometry (ESMS). No malonylation of *Sc.* FAS holo-ACP was observed in samples lacking C17S act holo-ACP (Figure 1A), confirming previous observations that FAS ACPs have no ability to self-malonylate. When incubated with 2, 6, and 10 μM C17S act holo-ACP, the extent of malonyl *S. coelicolor* FAS ACP (*Sc.*

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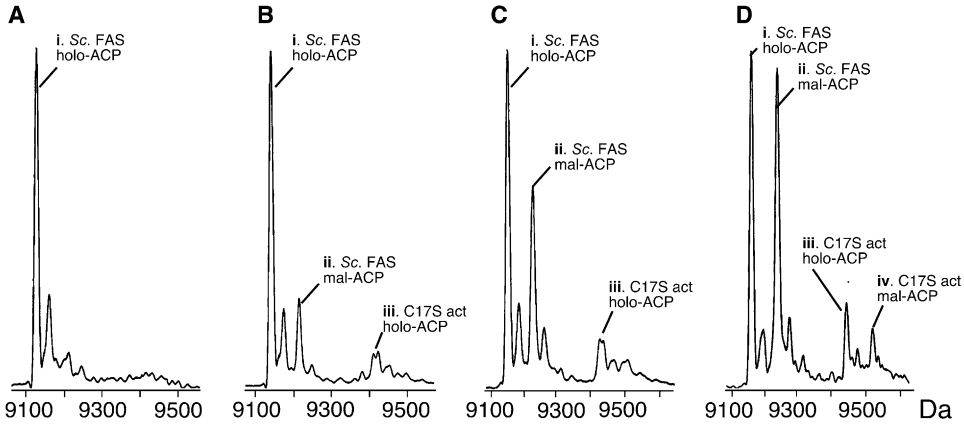

Figure 1. Act ACP-Catalyzed Transfer of a Malonyl Group to *Sc*. FAS holo-ACP

(A) negative control, (B) 2 μM act ACP, (C) 6 μM act ACP, and (D) 10 μM act ACP. Calculated mass values: (i) *Sc*. FAS holo-ACP (calculated 9125 Da, observed 9125 ± 1 Da), (ii) *Sc*. FAS mal-ACP (calculated 9211 Da, observed 9210 ± 2 Da), (iii) C17S act holo-ACP (calculated 9442 Da, observed 9440 ± 1 Da), and (iv) C17S act mal-ACP (calculated 9525 Da, observed 9527 ± 1 Da).

FAS mal-ACP) formation was found to be dependent on the PKS ACP concentration (Figures 1B–1D). When act ACP was used in the apo form, no malonyl transfer to *Sc*. FAS holo-ACP was observed. Converting this apo-ACP to the holo form by using *S. coelicolor* holo-acyl carrier protein synthase (ACPS), however, immediately restored the acyl transfer ability of the carrier protein. We have previously shown that *S. coelicolor* ACPS has no malonyl transferase activity, confirming that only holo-ACP is responsible for the observed malonyl transfer activity [14]. Replacing act ACP with another type II polyketide ACP (either wild-type [no C17S mutation; WT act holo-ACP] or *S. rimosus* oxytetracycline holo-ACP [otc holo-ACP]) [16] or interchanging the FAS ACP with other FAS ACPs (*E. coli* or the type I rat ACP domain) gave similar levels of malonyl transfer (data not shown). If malonylated *S. coelicolor* actinorhodin C17S ACP (C17S act mal-ACP) was preformed by incubation of C17S act apo-ACP with ACPS and malonyl CoA, followed by gel filtration, then transfer to both *S. coelicolor* and *E. coli* FAS ACPs was also observed. Transfer was limited to malonate as a substrate. When acetyl, acetoacetyl, or butyryl act ACPs were substituted for malonyl ACP, no transfer was observed.

To test for transfer of malonate between polyketide ACPs, otc and act ACPs were utilized. The self-malonylation properties of both polyketide ACPs [10] prevented the use of malonyl CoA and required that one polyketide component be preloaded with a malonyl group. In the first example, WT act mal-ACP was used to transfer its malonyl group to otc holo-ACP. Again, transfer was measured by ESMS (Figure 2A). The reciprocal assay with malonyl otc ACP (otc mal-ACP) and C17S act holo-ACP is shown in Figure 2B. In both cases, malonyl transfer was observed between the PKS ACPs giving otc mal-ACP (55%) and C17S act mal-ACP (65%) relative to their holo counterparts. Lastly, we used a ^{15}N-labeled sample of C17S act mal-ACP to investigate transfer of malonate to unlabeled C17S act holo-ACP. Figure 2C shows a time course with samples collected at 0, 5, and 15 min and analyzed by ESMS. At 0 min, ^{15}N C17S act mal-ACP and C17S act holo-ACP are clearly the predominant species, although a small amount of ^{15}N C17S act holo-ACP is present due to cleavage of the thiolester and loss of malonate. At 5 and 15 min, however, there is a clear accumulation of C17S act mal-ACP and an increase of ^{15}N C17S act holo-ACP, indicating successful malonyl transfer.

We next explored whether the type II FAS ACPs were capable of transferring malonate to another ACP. To test FAS-to-FAS ACP transfer, preformed *Sc.* FAS mal-ACP was incubated with *E. coli* FAS holo-ACP. ESMS analysis of this reaction revealed no transfer of the malonyl group (two peaks only observed after 60 min; *E. coli* FAS holo-ACP [minus N-terminal methionine] [calculated 8849 Da, observed 8848 ± 2 Da], *Sc*. FAS mal-ACP [calculated 9211 Da, observed 9212 ± 2 Da]). Transfer from *Sc*. FAS mal-ACP to a second *Sc.* FAS holo-ACP was also tested. To achieve this, *Sc.* FAS mal-ACP was uniformly ^{15}N labeled and therefore distinguishable by ESMS. ESMS analysis of this reaction again revealed no formation of unlabeled *Sc*. FAS mal-ACP, confirming that there was no transfer of the malonyl group (two peaks only observed after 60 min; *Sc*. FAS ACP [calculated 9125 Da, observed 9126 ± 1 Da], ^{15}N *Sc*. FAS mal-ACP [calculated 9307 Da, observed 9302 ± 2 Da]). When *Sc*. FAS mal-ACP was incubated with C17S act holo-ACP (testing FAS-to-PKS ACP transfer), again no transfer of malonate was observed (data not shown).

When we incubated N-acetylcytsteamine with C17S act mal-ACP, we found no evidence of transfer from the protein bound prosthetic group to this free, low-molecular weight thiol-bearing molecule (data not shown). We found no evidence of the decrease in the levels of C17S act mal-ACP over the time course of the experiment, nor did we detect any formation of malonylated N-acetylcysteamine.

Influence of Point Mutations on the Malonyl Transferase (Donor) Activity of C17S Act Holo-ACP

To probe the potential role of arginine residues in ACP-ACP transacylation, R11A, R34A, and R72A mutants of C17S act ACP were constructed, expressed in *E. coli*, and purified. The N79A mutation was also included since N79 has high sequence conservation in type II PKS

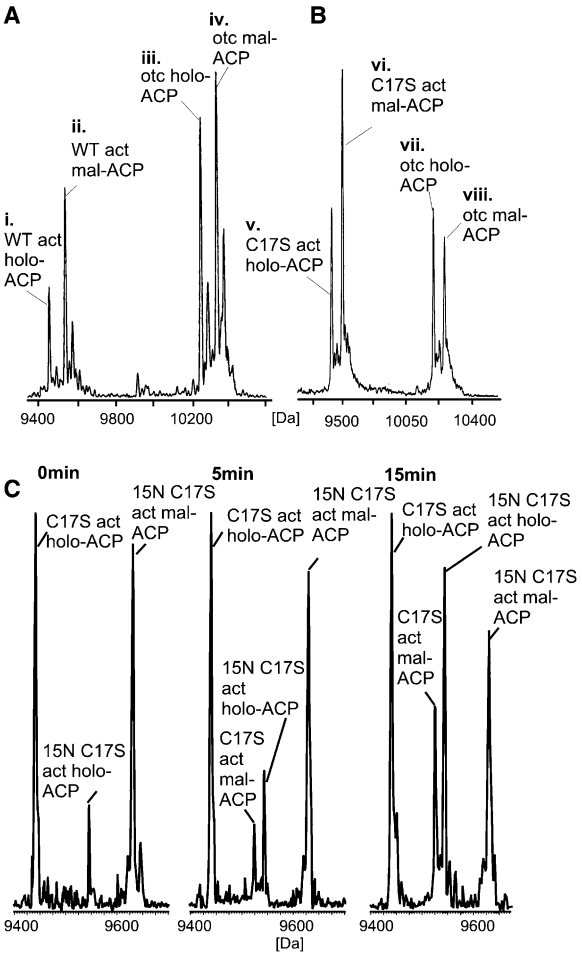

Figure 2. Malonyl Transfer between Polyketide ACPs

(A) Transformed ESMS of WT act mal-ACP incubated with otc holo-ACP after 30 min.

(B) Transformed ESMS of otc mal-ACP incubated with C17S act holo-ACP for 30 min. (i) WT act holo-ACP (calculated 9455 Da, observed 9454 ± 2 Da). (ii) WT act mal-ACP (calculated 9541 Da, observed 9541 ± 1 Da). (iii) otc holo-ACP (calculated 10256 Da, observed 10254 ± 2 Da). (iv) otc mal-ACP (calculated 10342 Da, observed 10340 ± 2 Da). (v) C17S act holo-ACP (calculated 9442 Da, observed 9440 ± 1 Da). (vi) C17S act mal-ACP (calculated 9525 Da, observed 9525 ± 1 Da). (vii) otc holo-ACP (calculated 10256 Da, observed 10255 ± 2 Da). (viii) otc mal-ACP (calculated 10342 Da, observed 10341 ± 2 Da).

(C) Transformed ESMS of ^{15}N act mal-ACP incubated with unlabeled C17S act holo-ACP.

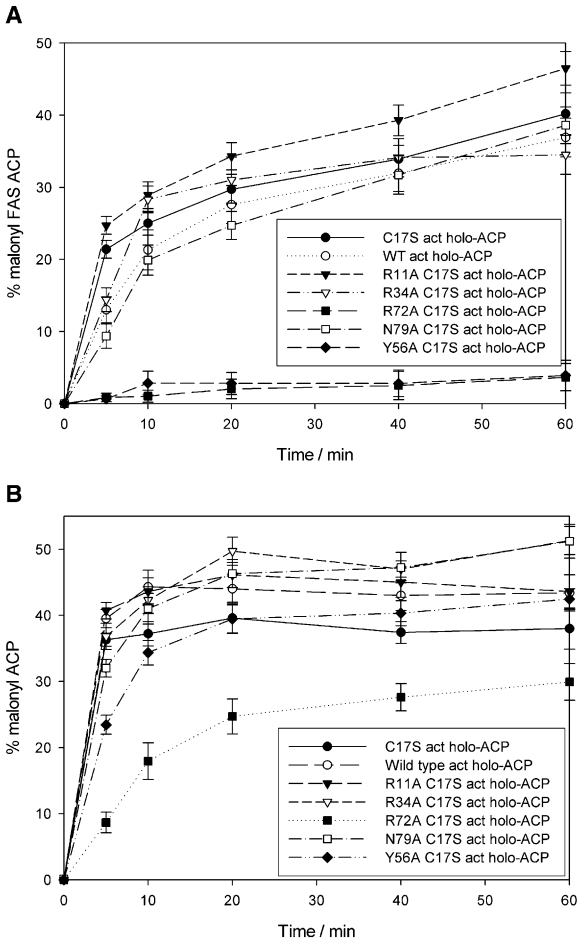

Figure 3. Malonyl Transfer and Self-Malonylation of the C17S R11A, R34A, R72A, N79A, and Y56A Mutants

(A) Percentage of malonyl transfer to *Sc.* FAS holo-ACP from malonyl CoA with PKS ACP mutants.

(B) Percentage of self-malonylation of PKS ACP mutants from malonyl CoA. The extent of acylation was calculated from the relative peak areas of the transformed electrospray mass spectra. Assays were performed in triplicate, and error bars represent 95% confidence intervals.

ACPs [17]. Successful ACP mutagenesis was confirmed by DNA sequencing and ESMS (R11A, calculated 9355 Da, observed 9356 ± 1 Da; R34A, calculated 9355 Da, observed 9355 ± 1 Da; R72A, calculated 9355 Da, observed 9356 ± 2 Da; N79A, calculated 9399 Da, observed 9398 ± 1 Da). Malonyl transferase and self-malonylation activities of all RXA and N79A act ACPs were established in identical assays to those for act ACP. With the exception of R72A, the malonyl transfer activity of all of the above-mentioned mutant ACPs was essentially identical to that of C17S act holo-ACP. The R72A mutation significantly impaired the ability of the C17S act holo-ACP to transfer malonate, with only 4% malonyl *Sc.* FAS ACP being formed compared to 25% in the standard act assay over the course of 10 min (Figure 3A). After 10 min, there was no further increase in malonyl transfer for the R72A mutant, while C17S act holo-ACP-catalyzed transfer rose to 40% over the next 50 min. Furthermore, R72A reduced the ability of the ACP to self-malonylate, giving only 17% malonylation after 10 min compared to 37% for C17S act holo-ACP (Figure 3B). The remaining mutants underwent self-malonylation to the maximum extent (40%–50%) after 10 min, but R72A continued to rise slowly, approaching 28% after 60 min.

2D NMR studies were performed to check for structural perturbations in the R72A C17S act ACP mutant. Chemical shifts were assigned by using a combination of ^{1}H-^{1}H 2D TOCSY and NOESY experiments and were then interpreted by using the chemical shift index (CSI) [18]. The CSI provides a robust method of interpreting protein chemical shifts in terms of secondary structure, particularly when comparing homologous proteins. The 3D structure of WT act apo-ACP and the CSI of WT and

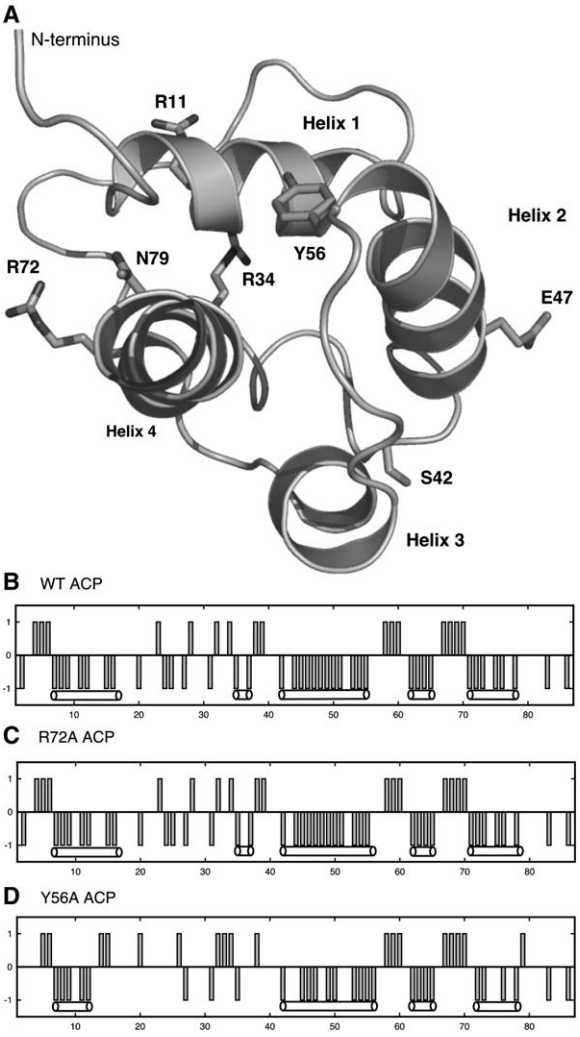

Figure 4. 3D Structure of WT Act Apo-ACP and Comparison of the Chemical Shift Indices of Act Apo-ACP Single Point Mutants

(A) Cartoon representation of the 3D structure of WT act apo-ACP. Helices 1–4 are clearly marked, and residues chosen for mutation are shown. Ser42, which bears the phosphopantetheine arm in the holo form of the ACP, is also shown.

(B–D) Comparison of the chemical shift index between (B) WT act ACP, (C) R72A C17S act ACP, and (D) Y56A C17S act ACP. Consecutive helical segments (CSI = −1) are represented as cylinders.

R72A C17S act holo-ACP are shown in Figure 4. As expected, the CSI for WT act holo-ACP showed a clear demarcation of the four major helices (Figure 4B) [17]. The CSI for R72A was essentially identical, suggesting a homologous secondary structure (Figure 4C). The ^{1}H-^{1}H NOESY spectrum was also used to identify key long range nOes [17, 19]. All key long-range nOes present in the WT act holo-ACP spectra could be identified in the R72A spectrum, suggesting that the detailed 3D structure was also preserved.

Influence of Point Mutations on the Malonyl Transferase (Acceptor) Activity of C17S Act Holo-ACP and *Sc.* FAS Holo-ACP

A number of studies have suggested that several negatively charged, solvent-exposed amino acids within helix II of FAS ACPs may have important roles in protein-protein interactions [20–22]. E47 in act ACP is highly conserved amongst the type II PKS ACPs, is also situated on helix II, and is directly analogous to those identified in the FAS systems. Malonyl transferase and self-malonylation activities of E47A C17S act ACP were established by using the same protocols used in mixed PKS/FAS or PKS/PKS ACP assays. Premalonylated C17S act ACP could transfer malonylate to E47A C17S act holo-ACP, but only at ~50% of the rate of a control sample that assayed transfer from ^{15}N C17S act mal-ACP to C17S act holo-ACP (Figure 5). E47A C17S act holo-ACP retained the ability to self-malonylate (data not shown) and could transfer malonate to either FAS or PKS carrier proteins at the same level as C17S act holo-ACP (Figure 5). When transfer from C17S act mal-ACP to E46A *Sc*. FAS holo-ACP was measured, transfer was more significantly reduced, yielding only 20% that of WT *Sc*. FAS holo-ACP (data not shown) over a 15 min assay. As expected, E46A *Sc*. FAS holo-ACP did not self-malonylate and could not transfer malonate to C17S act or *Sc*. FAS holo-ACP (data not shown).

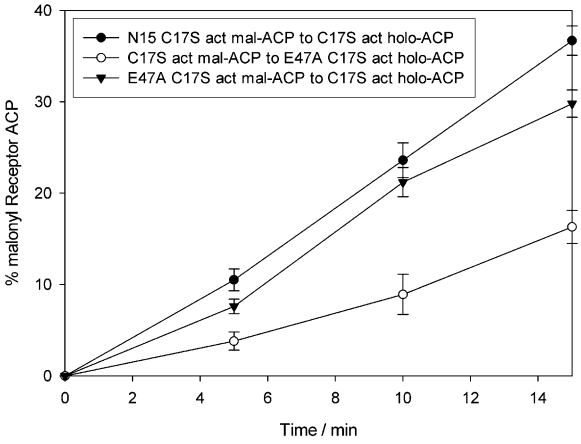

Figure 5. Malonyl Transfer and Acceptance of E47A C17S Act Mal-ACP and E47A C17S Act Holo-ACP

The extent of acylation was calculated from the relative peak areas of the transformed electrospray mass spectra. The control C17S act mal-ACP to C17S act holo-ACP transfer was performed by using ^{15}N-labeled C17S act mal-ACP. Assays were performed in triplicate, and error bars represent 95% confidence intervals.

Generation of a Malonyl-Transferring FAS ACP

It has been shown previously that the F50A mutation in *Vibrio harveyi* FAS ACP led to radical loss of the native ACP conformation and a reduction in noncovalent acyl chain binding [22]. We mutated the homologous residue in act ACP, Y56, to an alanine in order to determine whether this residue had a similar disruptive effect on act ACP structure. ACP mutagenesis was confirmed by DNA sequencing and ESMS (Y56A; calculated 9349 Da, observed 9350 ± 1 Da). While Y56A had no effect on self-malonylation, the ACP's ability to transacylate was reduced to one-tenth of control levels (Figures 3A and 3B). Comparison of the TOCSY and NOESY spectra for WT and Y56A ACPs showed significant differences, with helix I curtailed and helices II and IV showing less helical character (Figure 4D). We also observed the loss of several key long-range nOes to helix I and concomitant loss in helix I/helix IV nOes. Although the 3D

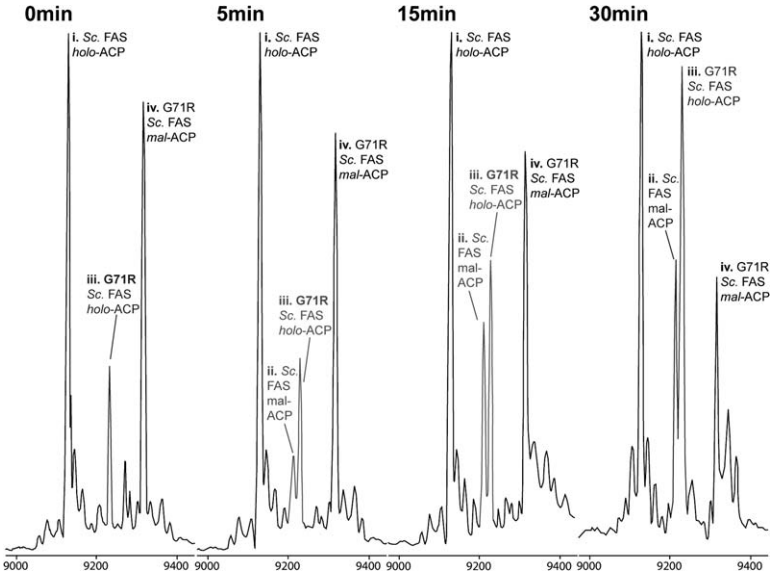

Figure 6. ESMS Analysis of Transfer Ability of G71R *Sc*. FAS ACP

Transfer of malonate from G71R *Sc*. FAS mal-ACP to *Sc*. FAS holo-ACP after 0, 5, 15, and 30 min. (i) *Sc.* FAS holo-ACP (calculated 9125 Da, observed 9124 ± 1 Da). (ii) *Sc.* FAS mal-ACP (calculated 9211 Da, observed 9211 ± 2 Da). (iii) G71R *Sc*. FAS holo-ACP (calculated 9225 Da, observed 9226 ± 1 Da). (iv) G71R *Sc.* FAS mal-ACP (calculated 9311 Da, observed 9313 ± 1 Da).

structure is significantly disrupted, the retention of self-malonylation and the loss of malonyl transfer imply that these activities can be decoupled. This led us to suggest that a nontransferring, non-self-malonylating ACP could be converted to one with either, or both, of these activities after a predictable mutation. Despite strong sequence and structural similarity with the act PKS ACP, the *Sc*. FAS ACP (unpublished structure) neither self-malonylates nor transacylates. Sequence alignment of this carrier protein with the PKS equivalent identified homology between G71 of *Sc*. FAS ACP and R72 of the act ACP. Thus, the G71R mutation was made in *Sc.* FAS ACP, and the protein was expressed, purified, and converted to the holo form. Initially the ability of G71R *Sc.* FAS holo-ACP to self-malonylate was assayed. Incubation of G71R *Sc*. FAS holo-ACP with an equivalent amount of malonyl CoA resulted in no observable malonylation of the ACP (data not shown). To test for transfer, G71R *Sc*. FAS mal-ACP was formed from G71R *Sc*. FAS apo-ACP by using malonyl CoA and ACPS (calculated 9311 Da, observed 9312 ± 1 Da). Then, G71R *Sc.* FAS mal-ACP and *Sc.* FAS holo-ACP were incubated together under standard assay conditions. Analysis by ESMS showed an approximate 30% transfer of malonate to *Sc*. FAS holo-ACP over 30 min (calculated 9211 Da, observed 9212 ± 2 Da) (Figure 6). This value is slower than, yet comparable to, transfer by act C17S holo-ACP.

Discussion

The observation of self-malonylation and malonyl transfer reactions of ACPs provides a possible explanation for the singular lack of MCAT genes in type II PKS clusters. The recent observation that a chemically synthesized ACP can undergo self-malonylation has reaffirmed this as a true property of type II PKS ACPs and categorically ruled out the possibility of MCAT contamination. The discovery of malonyl transfer has also been reported by others [12], although this was subsequently retracted [13], once again being incorrectly attributed to MCAT contamination. We have now confirmed that act ACP does indeed catalyze the transfer of the malonate moiety of malonyl CoA to *Sc*. FAS holo-ACP and other type II and type I ACPs; thus, it must also be considered to be an intrinsic activity of this protein.

The presence of the phosphopantetheine thiol in the holo form of the ACP is essential for malonyl transfer and self-malonylation, as neither act C17S apo-ACP nor free N-acetylcysteamine catalyze either of these processes. Conversion of an inactive act C17S apo-ACP to the holo form by using ACPS restores the acyl transfer ability of this carrier protein. As with many PKS ACPs, WT act ACP has a second thiol at position 17, though substitution of this thiol with a serine had no effect on the transfer activity. The implication of these findings is that PKS ACPs may transfer malonate from malonyl CoA to other ACPs through the process of self-malonylation followed by transfer to another ACP. We have also observed that premalonylated PKS ACPs are able to transfer malonate, indicating that the presence of free malonyl CoA is not an absolute requirement for catalysis to occur. Acyl transfer by MCAT shows high substrate specificity and is limited to malonate. Similarly, acyl transfer by act C17S ACP shows a similar high substrate specificity, with malonate being the only acyl group transferred. In contrast, self-acylation has a more relaxed selectivity. Malonyl, methylmalonyl, and acetoacetyl CoAs were observed to self-load with high yield, whereas butyryl and acetyl both failed [10]. A wide range of β-keto thiolesters in the form of their NAC thioesters have also been observed to self-acylate [11].

FAS ACPs do not catalyze acyl transfer to either FAS or PKS ACPs. While act PKS holo-ACP can transfer a malonyl group to a recipient *Sc.* FAS holo-ACP, *E. coli* FAS holo-ACP, or rat FAS holo-ACP domain, the reverse transfer from a FAS ACP to a PKS ACP was not observed. The otc PKS ACP [23], structurally homologous to act ACP [17], also transferred malonyl to the same FAS ACPs in a unidirectional fashion. In assays containing homologous or heterologous type II PKS ACPs, malonyl transfer is observed to be bidirectional. Thus, PKS ACPs possess both the ability to donate as well as accept malonate, while FAS ACPs possess only the ability

to accept this acyl group. This suggests that while the overall 3D structure of PKS and FAS ACPs may be similar, distinct differences between these proteins must exist. Protein-protein interactions are implicit in the transfer process. This is confirmed in the experiment in which the accepting ACP is removed completely and substituted with only the thiol-bearing N-acetylcysteamine. In this case, no malonyl transfer is observed.

There is clearly a donor and acceptor mechanism for the observed transfer of malonate between acyl carrier proteins. We chose to investigate the possible role of arginine residues in the donor mechanism based on high sequence conservation in PKS ACPs, unusual chemical shifts of side chain protons [17, 19], and the fact that these basic residues have been implicated in mediating protein-protein interactions between *E. coli* FAS ACP and several discrete FAS enzymes [24]. When residues R11, R34, and R72 were mutated to alanine, only the R72A mutation had any affect on self-malonylation and malonyl transfer. R72 is 100% conserved in type II PKS ACPs and is known to be partially buried in the *act* [17], *fren* [25], and *otc* [23] structures. The possibility of a significant structural perturbation was discounted by performing a full NMR chemical shift assignment of the protein and a 2D ^{1}H-^{1}H NOESY analysis. The role of R72 in malonyl transfer is not clear. Our results show that the presence of R72 is important for self-malonylation and is required for malonyl transfer between ACPs. We do not understand what the role of this amino acid is, though R72 may be either involved in docking or mechanistically in the transfer process. We are currently conducting additional structure-function studies with the ACP and other PKS enzymes to identify the full complement of residues involved in molecular recognition, malonyl transfer, and self-malonylation.

Sc. FAS holo-ACP can act as an acceptor of malonyl groups, but it cannot catalyze transfer. Since the FAS protein is able to accept a malonyl group from a polyketide ACP, it must possess the necessary docking site for the malonyl PKS ACP. Sequence alignment and analysis of the crystal structure of *B. subtilis* ACP docked with ACPS identified a number of acidic residues that may mediate protein-protein interactions [20]. Introduction of the E46A mutation into *Sc.* FAS ACP severely impaired its ability to act as an acceptor of malonate, reducing the transfer of this acyl group to the FAS ACP by 80% over a 30 min assay. The E47A act ACP mutation had a less significant effect and reduced its ability to accept transfer of malonate by 50%. This suggests that these negatively charged residues may form part of the docking site for ACP-ACP interactions. The fact that the act E47A mutant only partially reduced transfer suggests that other residues may be important in ACP-ACP interactions. The E47A mutant of act ACP retained the ability to self-malonylate, suggesting that the acceptance mechanism and the self-malonylation mechanism are distinct.

It has been shown previously that the F50A mutation in *Vibrio harveyi* FAS ACP led to a significant loss of the native ACP conformation [22]. Mutating Y56 to alanine in C17S act ACP gave similar structural perturbations and proved crucial in discriminating between malonyl transfer and self-malonylation. This mutation resulted in a 10-fold decrease in ACP-catalyzed malonyl transfer over 10 min but had no effect on self-malonylation, again suggesting that these processes may occur through different mechanisms centered on separate regions of the protein.

Although self-malonylation and malonyl transfer are clearly not the same processes, the R72A mutation in the act ACP system fails to fully distinguish mechanistically between them. As *Sc*. FAS holo-ACP can neither self-malonylate nor transfer, we hypothesized that making the reciprocal G71R mutation could transform *Sc*. FAS holo-ACP into a protein with one or both of the self-malonylation or transferase properties of a PKS ACP. Our experiments demonstrated that the G71R mutation successfully converted a nontransferring *Sc*. FAS holo-ACP into one with the ability to transfer malonyl groups at a rate comparable to act holo-ACP. This mutation, however, did not confer the ability to self-malonylate to *Sc*. FAS holo-ACP. This agrees with our mutational data on act ACP that show that R72A only partially reduced self-malonylation. Clearly, for the act protein R72 must be only one of several residues implicated in this process. For transfer, however, the introduction of the G71R mutation either provides this single important residue for the *Sc*. FAS holo-ACP or completes a set of residues involved in the acyl transfer mechanism.

We have previously published in vitro kinetic parameters for self-malonylation of C17S act holo-ACP (K_M = 219 μM, k_{cat} = 0.34 min^{-1}) [10], *S. coelicolor* MCAT-catalyzed malonyl transfer to *S. coelicolor* FAS ACP (K_M = 73 μM [ACP], 60 μM [malonyl CoA], k_{cat} =450 s^{-1}) [26], and polyketide turnover by the minimal PKS (maximal rate of 650 pmol min^{-1} at 200 μM ACP) [27]. The malonyl transfer we describe in this paper provides in vitro evidence that ACP cannot only select malonyl CoA, but can also specifically transfer these malonyl groups (albeit at a rate several orders of magnitude slower than the MCAT). Thus, in an in vitro situation ACP acts as a malonyl transferase and is all that is required to produce polyketides in the presence of the other minimal components, KS_{α} and $KS\beta$.

The alternative to the lack of an MCAT gene is for *S. coelicolor* act PKS to recruit MCAT from FAS. Enzyme assays have suggested that MCAT levels can be determined during the growth and stationary phases of *S. coelicolor*, implying that MCAT may be present during polyketide biosynthesis [4]. These are, however, whole cell homogenates and do not indicate any subcellular compartmentalization that may be occurring and would prevent such enzyme crosstalk. Subsequently, however, extensive proteomic analysis of *S. coelicolor* during the stationary growth phase failed to detect MCAT [28, 29]. The work described in this paper addresses the lack of an MCAT gene in the PKS cluster and suggests that the malonyl transfer ability of an ACP could substitute for the lack of an MCAT enzyme, albeit at a much lower rate. This in vitro work may shed some light on the in vivo mechanism of polyketide production by the minimal PKS.

Significance

Despite similar overall 3D structures, the PKS and FAS type II acyl carrier proteins are required to transport very different chemical intermediates, interact with

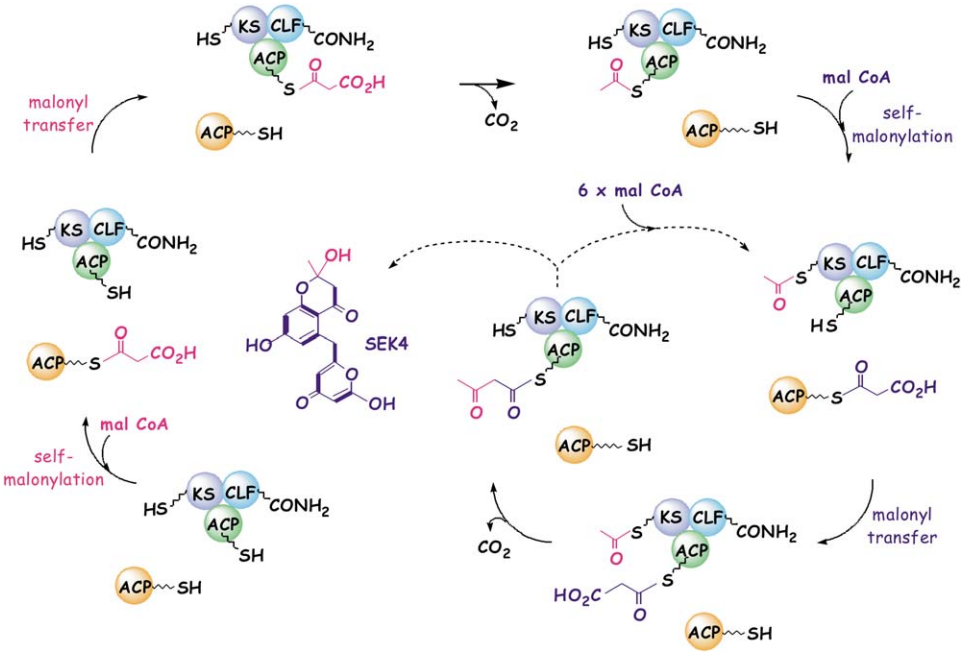

Figure 7. Schematic Representation of Type II Polyketide Biosynthesis in the Absence of MCAT

The minimal PKS (KS [purple], CLF [blue], and bound ACP [green]) interacts with free malonylated ACP (yellow). Progressing clockwise, malonate required for starter unit generation and chain elongation is continuously supplied to the bound ACP from a pool of free malonyl ACP.

different PKS or FAS components, and have a limited capacity to substitute for one another in vivo [30]. In addition, only PKS ACPs can undergo self-malonylation [10]. In this report, we have shown that these differences extend to another property, self-catalyzed malonyl transfer. Malonyl transfer is clearly a property of act ACP, though there remains the question of whether ACP-ACP malonyl transfer plays any role in polyketide production in vivo. In vitro we have shown that the minimal PKS consisting of just KS_α/KS_β and ACP is sufficient for polyketide production, and that if transfer occurs it does so fast enough to sustain this process [27]. The requirement for a continuous supply of malonyl ACP, the lack of a discrete MCAT analog, and the need to sequester a growing polyketide chain does therefore suggest several mechanistic possibilities. In the absence of a discrete malonyl transferase enzyme, the ACP-ACP transacylase activity may substitute for a dedicated malonyl transferase. In a previously proposed model for polyketide biosynthesis in the act minimal system [31], a priming malonyl ACP is first decarboxylated by KS_β to give acetyl ACP [32]. The ACP then translocates to KS_α and, once bound, delivers the acetyl group to the KS_α cysteine thiol. This ACP may then remain associated with the KS_α, and a second malonyl ACP binds to the complex and transfers its malonyl group to the bound ACP. The bound ACP remains associated with the KS_α through the first and subsequent rounds of decarboxylative condensation, being continually fed by ACP-ACP malonyl transfer (Figure 7). Such a mechanism eliminates the necessity for at least one of the ACPs to dissociate from the polyketide synthase complex, and by maintaining this complex, aberrant cyclization of the growing polyketide chain may be prevented [33].

Experimental Procedures

Chemical and Microbiological Reagents

Unless otherwise stated all chemicals were purchased from Sigma-Aldrich, UK. *E. coli* strains BL21 (DE3) and XL1-Blue were purchased from Novagen, Nottingham, UK and Stratagene, Cedar Creek, USA, respectively.

Protein Preparation and Purification

ACPs were expressed and purified by following the method of Crosby et al. [34]. The C17S mutant of act ACP was utilized in all of the in vitro PKS assays involving act ACP, as it has been shown that WT act holo-ACP can form an intramolecular disulphide bridge between the phosphopantetheine thiol and the thiol group of C17 [35]. ACPS was expressed and purified by following the method of Cox et al. [15]. Ketosynthase dimer (KS_α/KS_β) was purified as previously reported [27]. Where required, phosphopantetheinylation of apo-ACP to yield holo-ACP was achieved by incubation of purified apo-ACP (100 μM) with ACPS (1 μM) and coenzyme A (1 mM) in the presence of dithiothreitol (DTT, 5 mM, Melford Laboratories, Ltd., Ipswich, UK), $MgCl_2$ (10 mM), and Trizma base (TRIS-Cl, 50 mM [pH 8.8]) [36]. Reactions were incubated at 30ºC for 16 hr, and apo- to holo-ACP conversion was confirmed by ESMS. Prior to use, stock ACP samples (500 μM) were reduced and monomerized with DTT for 16 hr at 30ºC.

Preparation of Acyl ACPs

(1) Acetyl ACP was prepared by using C17S act holo-ACP (500 μM), EDTA (1 mM), N-acetyl imidazolide (4 mM), and potassium phosphate buffer (50 mM, pH 7.0). The reaction was stopped after 30 min, desalted into MilliQ water, and lyophilized. Formation of acetyl ACP was monitored by ESMS. (2) Malonyl ACP was prepared by using C17S act holo-ACP (500 μM) and malonyl CoA (5 mM) in potassium phosphate buffer (50 mM [pH 7.0]) and incubating for 6 hr. (3) Acetoacetyl ACP was prepared by using C17S act holo-ACP (500 μM), acetoacetyl *N*-acetylcysteamine (SNAC) thiolester [37] (30 mM), and potassium phosphate buffer (50 mM). (4) Butyryl ACP was prepared by using C17S act apo-ACP (500 μM), ACPS (0.25 μM), butyryl coenzyme A (750 μM), $MgCl_2$ (10 mM), and TRIS-Cl (50 mM [pH 8.8]). The conversion was carried out at 30ºC for 16 hr.

E. coli FAS holo-ACP (Sigma-Aldrich) was purified by anion exchange chromatography and treated with ammonium hydroxide (0.2 M [pH 7.0]) for 4 hr at 30ºC to remove any acyl adducts. Where required, ACP was labeled with ^{15}N by expression in M9 minimal media containing ^{15}N ammonium chloride as the sole source of nitrogen [15]. The ACP was purified by using standard techniques described above, and the percentage of label incorporated was determined by ESMS to be greater than 97%.

Electrospray Mass Spectrometry

Protein ESMS was performed in positive ion mode on a Fison's Instruments VG Quattro triple quadrupole mass spectrometer attached to a Jasco PU-980 Intelligent HPLC Pump operating at a 30 μl/min flow rate and a 50:50 v/v mixture of water and acetonitrile as the running solvent. Unless otherwise stated, all assays were quenched by mixing with C4 resin (Jupiter C4 100 Å, Phenomenex) in methanol and desalted prior to ESMS according to the method described by Winston and Fitzgerald [38].

Malonyl Transfer Activity of PKS and FAS ACPs

The effect on malonyl transfer of increasing amounts of C17S act holo-ACP was assayed by using *Sc.* FAS holo-ACP (50 μM), C17S act holo-ACP (0, 2, 6, 10 μM), DTT (0.1 mM), malonyl CoA (50 μM), and potassium phosphate buffer (50 mM [pH 7.0]). The mixture was incubated in a final volume of 50 μl for 30 min at 30ºC and then quenched as described above. For the control reactions, identical assays were carried out in which C17S act holo-ACP was replaced with C17S act apo-ACP. In order to confirm the malonyl transfer ability of holo-ACP, C17S act apo-ACP used in these control experiments was then converted to the holo form by using ACPS (described above), and the assay was repeated to test for restoration of malonyl transfer. In addition assays, were performed in which C17S act holo-ACP was substituted with WT act holo-ACP (no C17S mutation) and otc holo-ACP. *Sc*. FAS holo-ACP was also substituted with either the rat FAS holo-ACP domain or *E. coli* FAS holo-ACP. In these assays and those incorporating point mutations, the following reagents were used: standard FAS ACP (50 μM), DTT (0.1 mM), malonyl CoA (50 μM), and PKS ACP (1 μM), all in potassium phosphate buffer (50 mM [pH 7.0]) in a final volume of 50 μl. The assays were incubated at 30ºC for 15 or 30 min and then quenched. Assays were performed in triplicate.

Malonyl Transfer Ability of Malonyl *S. coelicolor* FAS ACP

Sc. FAS mal-ACP was first prepared by using *Sc*. FAS apo-ACP (50 μM), ACPS (1 μM), malonyl CoA (500 μM) in TRIS-HCl (50 mM [pH 8.8]) and $MgCl_2$ (10 mM) buffer. The reaction was incubated at 30ºC for 2 hr, at which point the reaction was desalted into MilliQ water, flash frozen, and lyophilized. ESMS of the sample indicated a small amount of *Sc*. FAS holo-ACP, which was removed by allowing the holo-ACP to covalently dimerize and then by utilizing size exclusion column chromatography. *Sc*. FAS mal-ACP (50 μM) was then incubated with monomerized C17S act holo-ACP (50 μM) in potassium phosphate buffer (50 mM [pH 7.0], 50 μl) for 15 min at 30ºC. C17S act holo-ACP was also substituted with *E. coli* FAS holo-ACP and ^{15}N-labeled *Sc*. FAS holo-ACP.

Malonyl Transfer between PKS ACPs

Typical assays contained act WT or otc mal-ACP (50 μM) incubated with monomerized otc or C17S act holo-ACP (50 μM), respectively, in potassium phosphate buffer (50 mM [pH 7.0], 50 μl) for 30 min at 30ºC. Reactions were quenched and analyzed by ESMS. The transfer of malonate from one molecule of act ACP to another was monitored by observing the transfer of malonate from ^{15}N C17S act mal-ACP to unlabeled C17S act holo-ACP.

Transfer of Other Acyl Groups between ACP Species

Sc. FAS holo-ACP (50 μM) and C17S act acyl ACP (10 μM) were incubated together for up to 2 hr in potassium phosphate buffer (50 mM, 50 μl [pH 7.0]) and then quenched. Displacement of acetyl/butyryl groups by malonyl CoA was assayed by incubating act acetyl/butyryl ACP (50 μM), malonyl CoA (1 mM), and potassium phosphate buffer (50 mM, 50 μl [pH 7.0]) for 2 hr at 30ºC.

Transfer of Acyl Groups between C17S Act Malonyl ACP and N-Acetylcysteamine

C17S act mal-ACP (50 μM) was incubated with N-acetylcysteamine (50 μM) in potassium phosphate buffer (50 mM [pH 7.0], 50 μl) for 30 min at 30ºC. Reactions were quenched and analyzed by ESMS to detect malonylated N-acetylcysteamine and an increase in C17S act holo-ACP.

Site-Directed Mutagenesis and Expression of Act R11A, R34A, and R72A ACP Mutants

Divergent polymerase chain reaction (PCR) was carried out by using pRJC001 [15] (coexpressing the C17S act ACP and *E. coli* ACPS in the plasmid pT7-7). PCR reactions were carried out by using VENT proof-reading polymerase under the following conditions (annealing, elongation, denaturing): R11A: 96ºC, 60 s; 65ºC, 75 s; 72ºC, 165 s; R34A: 96ºC, 60 s; 63ºC, 60 s; 72ºC, 180 s; R72A: 96ºC, 60 s; 65ºC, 75 s; 72ºC, 165 s, for 20 cycles in each case. Purified DNA was digested with DpnI and transformed into *E. coli* DH5α. Individual clones were selected, and their plasmid DNA was isolated and sequenced. R11A and R34A act ACPs were expressed in the holo form from *E. coli* K38 pGP1-2 [39]. R72A act ACP was ligated into pET11c vector and was expressed in the apo form in *E. coli* BL21 (DE3). The resultant apo protein was converted to the holo form by using ACPS as described previously [15].

Cloning and Expression of *S. coelicolor* Y40A, E47A, Y56A, and N79A of C17S ACPs and G71R and E46A *Sc.* FAS ACPs

Site-directed mutagenesis of act ACP was performed on plasmid pCJAI159/1F (a coexpression plasmid encoding for act ACP and *E. coli* ACPS [40]) by using the QuikChange site-directed mutagenesis kit (Stratagene) following the manufacturer's protocol. The oligonucleotide primers (Table S1; see the Supplemental Data available with this article online) were synthesized (Lark Technologies, Cambridge) and purified by polyacrylamide gel electrophoresis. dsDNA template (50 ng) was used as standard. The plasmids isolated from XL1-Blue were transformed into *E. coli* BL21 (DE3) and maintained as suspensions in 20% glycerol at −80ºC. Proteins were expressed and purified as described above. Site-directed mutagenesis of *Sc*. FAS ACP was performed on plasmid pIJ8130 (a pET-based expression plasmid encoding for *Sc*. FAS ACP).

Effect of G71R Mutagenesis on Malonyl Transfer

Typical assays contained G71R *Sc*. FAS mal-ACP (25 μM) incubated with reduced *Sc*. FAS holo-ACP (25 μM) in potassium phosphate buffer (50 mM [pH 7.0]) at 30ºC in a total volume of 50 μl. Assays were quenched at 0, 5, 15, and 30 min and were analyzed by ESMS.

NMR Analysis of Y56A and R72A Act ACPs

All NMR spectra were collected on a Varian *INOVA* 600 MHz spectrometer. Standard COSY, NOESY, and TOCSY data sets were acquired and compared to the equivalent 2D data sets for WT act apo-ACP [19].

Supplemental Data

Supplemental Data include a table detailing oligonucleotides used for site-directed mutagenesis and are available at http://www.chembiol.com/cgi/content/full/13/6/587/DC1/.

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References

1. Hopwood, D.A. (1997). Genetic contributions to understanding polyketide synthases. Chem. Rev. *97*, 2465–2497.
2. Rock, C.O., and Jackowski, S. (2002). Forty years of bacterial fatty acid synthesis. Biochem. Biophys. Res. Commun. *292*, 1155–1166.
3. Staunton, J., and Weissman, K.J. (2001). Polyketide biosynthesis: a millennium review. Nat. Prod. Rep. *18*, 380–416.
4. Revill, W.P., Bibb, M.J., and Hopwood, D.A. (1995). Purification of a malonyltransferase from *Streptomyces coelicolor* A3(2) and analysis of its genetic determinant. J. Bacteriol. *177*, 3946–3952.
5. Rock, C.O., and Cronan, J.E. (1996). *Escherichia coli* as a model for the regulation of dissociable (type II) fatty acid biosynthesis. Biochim. Biophys. Acta *1302*, 1–16.
6. Kremer, L., Nampoothiri, K.M., Lesjean, S., Dover, L.G., Graham, S., Betts, J., Brennan, P.J., Minnikin, D.E., Locht, C., and Besra, G.S. (2001). Biochemical characterization of acyl carrier protein (AcpM) and malonyl-CoA:AcpM transacylase (mtFabD), two major components of *Mycobacterium tuberculosis* fatty acid synthase II. J. Biol. Chem. *276*, 27967–27974.
7. Joshi, A.K., Witkowski, A., and Smith, S. (1998). The malonyl/acetyltransferase and β-ketoacyl synthase domains of the animal fatty acid synthase can cooperate with the acyl carrier protein domain of either subunit. Biochemistry *37*, 2515–2523.
8. Rawlings, B.J. (2001). Type I polyketide biosynthesis in bacteria (part B). Nat. Prod. Rep. *18*, 231–281.
9. Summers, R.G., Ali, A., Shen, B., Wessel, W.A., and Hutchinson, C.R. (1995). Malonyl-coenzyme A:acyl carrier protein acyltransferase of *Streptomyces glaucescens*: a possible link between fatty-acid and polyketide biosynthesis. Biochemistry *34*, 9389–9402.
10. Hitchman, T.S., Crosby, J., Byrom, K.J., Cox, R.J., and Simpson, T.J. (1998). Catalytic self-acylation of type II polyketide synthase acyl carrier proteins. Chem. Biol. *5*, 35–47.
11. Arthur, C., Cox, R.J., Crosby, J., Rahman, M.M., Simpson, T.J., Soulas, F., Spogli, R., Szafranska, A.E., Westcott, J., and Winfield, C.J. (2002). Synthesis and characterisation of acyl carrier protein bound polyketide analogues. ChemBioChem *3*, 253–257.
12. Zhou, P., Florova, G., and Reynolds, K.A. (1999). Polyketide synthase acyl carrier protein (ACP) as a substrate and a catalyst for malonyl ACP biosynthesis. Chem. Biol. *6*, 577–584.
13. Florova, G., Kazanina, G., and Reynolds, K.A. (2002). Enzymes involved in fatty acid and polyketide biosynthesis in *Streptomyces glaucescens*: role of FabH and FabD and their acyl carrier protein specificity. Biochemistry *41*, 10462–10471.
14. Arthur, C.J., Szfranska, A., Evans, S.E., Findlow, S.C., Burston, S.G., Owen, P., Clark-Lewis, I., Simpson, T.J., Crosby, J., and Crump, M.P. (2005). Self-malonylation is an intrinsic property of a chemically synthesized type II polyketide acyl carrier protein. Biochemistry *44*, 15414–15421.
15. Cox, R.J., Hitchman, T.S., Byrom, K.J., Findlow, I.S.C., Tanner, J.A., Crosby, J., and Simpson, T.J. (1997). Post-translational modification of heterologously expressed *Streptomyces* type II polyketide synthase acyl carrier proteins. FEBS Lett. *405*, 267–272.
16. Kim, E.S., Bibb, M.J., Butler, M.J., Hopwood, D.A., and Sherman, D.H. (1994). Sequences of the oxytetracycline polyketide synthase-encoding otc genes from *Streptomyces rimosus*. Gene *141*, 141–142.
17. Crump, M.P., Crosby, J., Dempsey, C.E., Parkinson, J.A., Murray, M., Hopwood, D.A., and Simpson, T.J. (1997). Solution structure of the actinorhodin polyketide synthase acyl carrier protein from *Streptomyces coelicolor* A3(2). Biochemistry *36*, 6000–6008.
18. Wishart, D.S., Sykes, B.D., and Richards, F.M. (1992). The chemical-shift index: a fast and simple method for the assignment of protein secondary structure through NMR spectroscopy. Biochemistry *31*, 1647–1651.
19. Crump, M.P., Crosby, J., Dempsey, C.E., Murray, M., Hopwood, D.A., and Simpson, T.J. (1996). Conserved secondary structure in the actinorhodin polyketide synthase acyl carrier protein from *Streptomyces coelicolor* A3(2) and the fatty acid synthase acyl carrier protein from *Escherichia coli*. FEBS Lett. *391*, 302–306.
20. Parris, K.D., Lin, L., Tam, A., Mathew, R., Hixon, J., Stahl, M., Fritz, C.C., Seehra, J., and Somers, W.S. (2000). Crystal structures of substrate binding to *Bacillus subtilis* holo-(acyl carrier protein) synthase reveal a novel trimeric arrangement of molecules resulting in three active sites. Struct. Fold. Des. *8*, 883–895.
21. Worsham, L.A.S., Earls, L., Jolly, C., Langston, K.G., Trent, M.S., and Ernst-Fonberg, M.L. (2003). Amino acid residues of *Escherichia coli* acyl carrier protein involved in heterologous protein interactions. Biochemistry *42*, 167–176.
22. Flaman, A.S., Chen, J.M., Van Iderstine, S.C., and Byers, D.M. (2001). Site-directed mutagenesis of acyl carrier protein (ACP) reveals amino acid residues involved in ACP structure and acyl-ACP synthetase activity. J. Biol. Chem. *276*, 35934–35939.
23. Findlow, S.C., Winsor, C., Simpson, T.J., Crosby, J., and Crump, M.P. (2003). Solution structure and dynamics of oxytetracycline polyketide synthase acyl carrier protein from *Streptomyces rimosus*. Biochemistry *42*, 8423–8433.
24. Zhang, Y.M., Rao, M.S., Heath, R.J., Price, A.C., Olson, A.J., Rock, C.O., and White, S.W. (2001). Identification and analysis of the acyl carrier protein (ACP) docking site on beta-ketoacyl-ACP synthase III. J. Biol. Chem. *276*, 8231–8238.
25. Li, Q., Khosla, C., Puglisi, J.D., and Liu, C.W. (2003). Solution structure and backbone dynamics of the holo form of the frenolicin acyl carrier protein. Biochemistry *42*, 4648–4657.
26. Szafranska, A.E., Hitchman, T.S., Cox, R.J., Crosby, J., and Simpson, T.J. (2002). Kinetic and mechanistic analysis of the malonyl CoA: ACP transacylase from *Streptomyces coelicolor* indicates a single catalytically competent serine nucleophile at the active site. Biochemistry *41*, 1421–1427.
27. Matharu, A.L., Cox, R.J., Crosby, J., Byrom, K.J., and Simpson, T.J. (1998). MCAT is not required for *in vitro* polyketide synthesis in a minimal actinorhodin polyketide synthase from *Streptomyces coelicolor*. Chem. Biol. *5*, 699–711.
28. Hesketh, A.R., Chandra, G., Shaw, A.D., Rowland, J.J., Kell, D.B., Bibb, M.J., and Chater, K.F. (2002). Primary and secondary metabolism, and post-translational protein modifications, as portrayed by proteomic analysis of *Streptomyces coelicolor*. Mol. Microbiol. *46*, 917–932.
29. Hesketh, A., and Chater, K.F. (2003). Evidence from proteomics that some of the enzymes of actinorhodin biosynthesis have more than one form and may occupy distinctive cellular locations. J. Ind. Microbiol. Biotechnol. *30*, 523–529.
30. Khosla, C., McDaniel, R., Ebertkhosla, S., Torres, R., Sherman, D.H., Bibb, M.J., and Hopwood, D.A. (1993). Genetic construction and functional-analysis of hybrid polyketide synthases containing heterologous acyl carrier proteins. J. Bacteriol. *175*, 2197–2204.
31. Bisang, C., Long, P.F., Cortes, J., Westcott, J., Crosby, J., Matharu, A.L., Cox, R.J., Simpson, T.J., Staunton, J., and Leadlay, P.F. (1999). A chain initiation factor common to both modular and aromatic polyketide synthases. Nature *401*, 502–505.
32. Simpson, T.J. (2000). *In vivo* and *in vitro* biosynthetic studies: understanding and exploiting natural pathways. In Biodiversity—New Leads for the Pharmaceutical and Agrochemical Industries, S.K. Wrigley, M.A. Hayes, R. Thomas, E.J.T. Chrystal, and N. Nicholson, eds. (Cambridge, UK: Royal Society of Chemistry), pp. 233–248.
33. Petkovic, H., Thamchaipenet, A., Zhou, L., Raspor, P., Waterman, P.G., and Hunter, I.S. (1999). Disruption of an aromatase/cyclase from the oxytetracycline gene cluster of *Streptomyces rimosus* results in production of novel polyketides with shorter chain lengths. J. Biol. Chem. *274*, 32829–32834.
34. Crosby, J., Sherman, D.H., Bibb, M.J., Revill, W.P., Hopwood, D.A., and Simpson, T.J. (1995). Polyketide synthase acyl carrier proteins from *Streptomyces*: expression in *Escherichia coli*, purification and partial characterization. Biochim. Biophys. Acta *1251*, 32–42.
35. Crosby, J., Byrom, K.J., Hitchman, T.S., Cox, R.J., Crump, M.P., Findlow, I.S.C., Bibb, M.J., and Simpson, T.J. (1998). Acylation of *Streptomyces* type II polyketide synthase acyl carrier proteins. FEBS Lett. *433*, 132–138.

36. Cox, R.J., Crosby, J., Daltrop, O., Glod, F., Jarzabek, M.E., Nicholson, T.P., Reed, M., Simpson, T.J., Smith, L.H., Soulas, F., et al. (2002). *Streptomyces coelicolor* phosphopantetheinyl transferase: A promiscuous activator of polyketide and fatty acid synthase acyl carrier proteins. J. Chem. Soc. Perkin Trans. *1*, 1644–1649.
37. Jacobs, A., Staunton, J., and Sutkowski, A.C. (1991). Aspyrone biosynthesis in *Aspergillus melleus*: identification of the intermediates formed on the polyketide synthase (PKS) in the 1st chain extension cycle leading to crotonate. J. Chem. Soc. Chem. Commun. 1113–1114.
38. Winston, R.L., and Fitzgerald, M.C. (1998). Concentration and desalting of protein samples for mass spectrometry analysis. Anal. Biochem. *262*, 83–85.
39. Tabor, S., and Richardson, C.C. (1985). A bacteriophage T7 RNA polymerase promoter system for controlled exclusive expression of specific genes. Proc. Natl. Acad. Sci. USA *82*, 1074–1078.
40. Arthur, C. (2005). *In vitro* Study of Protein: Substrate Interactions in the Actinorhodin Polyketide Synthase (Bristol, UK: University of Bristol).