Self-Malonylation Is an Intrinsic Property of a Chemically Synthesized Type II Polyketide Synthase Acyl Carrier Protein[†,‡]

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ABSTRACT: During polyketide biosynthesis, malonyl groups are transferred to the acyl carrier protein (ACP) component of the polyketide synthase (PKS), and it has been shown that a number of type II polyketide ACPs undergo rapid self-acylation from malonyl-CoA in the absence of a malonyl-CoA:holo-acyl carrier protein transacylase (MCAT). More recently, however, the observation of self-malonylation has been ascribed to contamination with *Escherichia coli* MCAT (FabD) rather than an intrinsic property of the ACP. The wild-type apo-ACP from the actinorhodin (act) PKS of *Streptomyces coelicolor* (synthetic apo-ACP) has therefore been synthesized using solid-state peptide methods and refolded using the GroEL/ES chaperone system from *E. coli*. Correct folding of the act ACP has been confirmed by circular dichroism (CD) and ^{1}H NMR. Synthetic apo-ACP was phosphopantetheinylated to 100% by *S. coelicolor* holo-acyl carrier protein synthase (ACPS), and the resultant holo-ACP underwent self-malonylation in the presence of malonyl-CoA. No malonylation of negative controls was observed, confirming that the use of ACPS and GroEL/ES did not introduce contamination with *E. coli* MCAT. This result proves unequivocally that self-malonylation is an inherent activity of this PKS ACP in vitro.

The genes required for the biosynthesis of polyketide secondary metabolites in *Streptomyces* are usually found clustered together (*1*). The actinorhodin (act)[1] cluster from *Streptomyces coelicolor*, for example, was thought to contain all of the genes (23) required for actinorhodin biosynthesis (*2*). This was confirmed by the observation of actinorhodin production on heterologous expression of the act gene cluster in *Streptomyces lividans* (*3*). Surprisingly, however, there appeared to be no discrete gene for a malonyl-CoA:holo-acyl carrier protein transacylase (MCAT) and, therefore, no clear mechanism for starter or chain extender unit selection within this cluster. Subsequent cloning and sequencing of other type II PKS gene clusters confirmed their lack of a dedicated MCAT (*4*). This is in marked contrast with all type I fatty acid synthase (FAS) (*5*) and polyketide synthase (PKS) proteins which possess integral acyl transferase (AT)/MCAT domains (*6*), while the type II FAS protein complexes of bacteria and plants also possess a discrete MCAT (*7*−*9*).

A possible rationale for this was provided by the observation that MCAT was present during both exponential and stationary growth phases in *S. coelicolor*, and it was suggested that this malonyl transferase activity may be shared between FAS and PKS complexes. In this model MCAT catalyzes malonyl transfer to the respective acyl carrier proteins (ACPs) in both fatty acid and polyketide systems, a concept that became known as cross-talk (*10*, *11*). This has been extended to incorporate recruitment of other fatty acid synthase components in polyketide synthases that require but lack a ketoreductase, dehydratase, and enoylreductase (*12*).

It was originally shown that minimal PKS systems could be constructed in vivo from three genes, the ketosynthase

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[1] Abbreviations: ACP, acyl carrier protein; act, actinorhodin; ACPS, holo-acyl carrier protein synthase; CD, circular dichroism; CLF, chain length factor; ESMS, electrospray mass spectrometry; FAS, fatty acid synthase; KS, ketosynthase; MCAT, malonyl-CoA:holo-acyl carrier protein transacylase; NOE, nuclear Overhauser enhancement; PKS, polyketide synthase; C17S apo-ACP, recombinant C17S actinorhodin apo-ACP; C17S holo-ACP, recombinant C17S actinorhodin holo-ACP; SNAC, β-keto-*N*-acetylcysteaminyl thiol esters; synthetic apo-ACP, synthetic actinorhodin apo-ACP; synthetic holo-ACP, synthetic actinorhodin holo-ACP.

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(KS), chain length factor (CLF), and ACP (*13*, *14*). This minimal system controlled starter unit selection, chain length, and the first cyclization of the nascent polyketide chain. In vitro, relatively crude preparations of KS/CLF and purified holo-ACPs [frenolicin (fren), granaticin (gra), oxytetracycline (otc), and tetracenomycin (tcm)] were shown to support polyketide biosynthesis (*15*, *16*). If KS/CLF preparations were purified to 95% homogeneity, however, and supplied with the same holo-ACPs, no polyketides were produced. Production was restored when a crude extract from a *S. coelicolor* strain that lacked the entire act PKS gene cluster was added to this preparation. The missing factor was purified and shown to be the FabD MCAT. This was seen to be further proof of the concept of cross-talk.

An alternative to the lack of an MCAT gene in type II clusters and, therefore, the necessity for cross-talk is for one of the minimal enzymes to have a dual functionality. A putative AT domain (GHSXG) was originally identified as part of the act KS (*17*), but mutagenesis of the active site serine showed that this amino acid was unnecessary for polyketide biosynthesis, ruling this motif out as the site of acyl transfer (*18*). The third component of the minimal system, the ACP, has the primary role of covalently binding malonate and the subsequent polyketide intermediates. In the course of studies of rigorously purified act PKS holo-ACP, we observed that it could undergo rapid acylation with malonate derived from malonyl-CoA in the absence of MCAT. This ability was shown to be shared by other type II PKS ACPs (*19*). The ACP seemed to possess inherent malonyl transfer activity, and this self-acylation reaction could be extended to other dicarboxylic and β-keto acids as either their CoA or *N*-acetylcysteamine thiol esters (*19*, *20*). Essentially, ACP appeared to be able to function as an acyl transferase but one with a broader specificity than MCAT itself. Self-malonylation was not observed in the corresponding type II FAS ACPs (*19*, *21*). It was subsequently demonstrated that in vitro a truly minimal PKS consisted of only the ACP and KS/CLF in the presence of malonyl-CoA with no requirement for a separate acyl transferase (*21*) and so provided a possible rationale for the lack of an MCAT gene in type II PKS gene clusters. A similar phenomenon has also been observed for the tcm synthase ACP (*22*, *23*). More recently, however, the observation of self-malonylation in the tcm ACP has been ascribed to contamination with *Escherichia coli* FabD. This was based on the observation that repeated chromatographic purification or production of the ACP in a baculovirus expression system (that does not possess a discrete MCAT) reduced or eliminated self-malonylation (*24*) and reestablished cross-talk as the primary means of substrate selection in systems where an acyl transferase is absent. However, it failed to account for our previous observation of self-acylation occurring with CoA and β-keto-*N*-acetylcysteaminyl thiol esters (SNAC) which cannot act as substrates for MCAT.

We now report studies on the structural and biochemical characterization of chemically synthesized act ACP. Our results show unequivocally that this ACP undergoes malonylation in the presence of malonyl-CoA alone. This categorically proves that self-malonylation is an inherent property of the acyl carrier protein and cannot be attributed to MCAT contamination.

MATERIALS AND METHODS

Chemical Synthesis of Wild-Type (WT) Apo-ACP. WT actinorhodin apo-ACP (synthetic apo-ACP) was synthesized using solid-phase methods that were optimized and adapted to a fully automated peptide synthesizer (Applied Biosystems 430A). Synthesis was started with the protected C-terminal amino acid (Ala) linked to a cross-linked polystyrene resin via a 4-(carboxamidomethyl)benzyl ester linkage (pam resin; Applied Biosystems) [0.3 mmol of 0.74 mmol/g pam resin (Applied Biosystems) was used]. N^{α}-*t*-Boc amino acids with the appropriate side chain protecting groups were added in a stepwise fashion until the entire polypeptide was formed. Side chain protection was as follows: benzyl (Asp, Glu, Ser, Thr); 4-methylbenzyl (Cys); toluenesulfenyl (Arg); and none (Ala, Asn, Gly, Ile, Leu, Met, Phe, Pro, Val). Samples were automatically taken after each step to retrospectively monitor the amino acid coupling yields using a ninhydrin-based reaction (*25*). Amino acids were purchased from Peptides International, Louisville, KY. The synthesis reagents *N*,*N*-diisopropylethylamine, 1-hydroxybenzotriazole, and dicyclohexylcarbodiimide were obtained from Applied Biosystems. Peptides were deprotected and cleaved from resin using hydrogen fluoride (Matheson Gas Products) with *p*-cresol and 4-methylbenzenethiol (Sigma-Aldrich) as scavengers (0 °C for 1 h). The polypeptide was purified by HPLC using 22 mm × 250 mm preparative and 10 mm × 250 mm semipreparative C18 reverse-phase columns (Vydac). Purity was confirmed with analytical HPLC [4.6 mm × 250 mm C18 reverse-phase column (Vydac)] and Mass spec (Sciex API 3 mass spectrometer). In total, 18.2 mg of synthetic apo-ACP was isolated.

Protein Purification. S. coelicolor PKS and FAS ACPs, KS/CLF, MCAT, and ACPS were purified using methods described previously (*21*, *26*−*28*).

GroEL/ES was purified using a slightly modified method from that described previously (*29*). GroEL and GroES were coexpressed in *E. coli* DH5α cells from the Trc99a plasmid. Cells were cultured in LB media supplemented with carbenicillin (100 μg/mL) at 37 °C until the optical density at 600 nm approached 0.8, at which point the cells were induced by the addition of IPTG to a final concentration of 1 mM and then harvested after a further 4 h at 37 °C. The cells were lysed by sonication in 50 mM Tris-HCl (pH 8.5) and 1 mM phenylmethanesulfonyl fluoride (PMSF), and the cell debris was removed by centrifugation. Nucleic acids were removed by precipitation with streptomycin sulfate (2 g/100 mL of cell-free extract) and clarified by centrifugation. The supernatant was loaded onto a 26/10 Q-Sepharose anion-exchange FPLC column (Amersham Pharmacia) equilibrated in 50 mM Tris-HCl (pH 8.5). The column was washed with 2 column volumes of 50 mM Tris-HCl (pH 8.5) before eluting over an 8 column volume gradient from 0 to 1 M NaCl. GroES eluted at approximately 400 mM NaCl, while GroEL eluted at 600 mM NaCl. GroEL-containing fractions were pooled, and HPLC-grade methanol was added to 25% (v/v) before application to an Sephadex 200 HR FPLC gel filtration column preequilibrated in 50 mM Tris-HCl (pH 8.5) and 25% (v/v) HPLC-grade methanol. GroEL-containing fractions were pooled and dialyzed against 50 mM Tris-HCl (pH 8.5) before being stored as a precipitate in 70% saturated ammonium sulfate. Likewise, fractions containing GroES

were pooled and dialyzed against 50 mM Tris-HCl (pH 8.5) before being stored as a precipitate in 70% saturated ammonium sulfate. The purity of GroEL and GroES was assessed by measurement of Trp fluorescence (GroEL/ES contain no tryptophans) and was found to contain less than 1 Trp residue per 50 GroEL or GroES monomers (*30*).

HPLC of Acyl Carrier Proteins. HPLC analysis was performed using a Jupiter C8 reverse-phase column, 250 × 4.6 (Phenomenex), on a Gilson Instruments HPLC. The column was equilibrated for 5 min after injection of the sample with 95% water, 5% acetonitrile, and 0.05% (v/v) trifluoroacetic acid (TFA) at a flow rate of 1 mL/min. The concentration of acetonitrile was then linearly increased to 95% over 30 min. Proteins were detected by monitoring the absorbance at 280 nm. A pure act apo-ACP standard was found to elute at approximately 16 min.

Electrospray Mass Spectrometry (ESMS). ESMS was performed 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 using a 50:50 water:acetonitrile mixture as the running solvent. Proteins were analyzed in positive ion mode with a cone voltage of 25 V. All samples were prepared as described by Winston and Fitzgerald (*31*).

Circular Dichroism (CD). CD spectra were recorded on an ISA Yobin Yvon CD6 dichrograph. All spectra were recorded at an ACP concentration of 1 mg/mL in potassium phosphate buffer (50 mM, pH 7.3) at 25 °C.

NMR Spectroscopy. All NMR samples were prepared in 20 mM K_2HPO_4 and 1 mM NaN_3, pH 5.5, with 5% D_2O, and spectra were acquired at 25 °C (*32*, *33*). Standard one-dimensional NMR experiments were acquired on a 600 MHz Varian INOVA spectrometer using WATERGATE for suppression of the residual water signal. All spectra were recorded with 32000 complex points and a spectral width of 10 kHz.

Protein Folding. (A) Urea/Stepwise Dilution. Lyophilized synthetic apo-ACP (3 mg) was dissolved in urea (8 M, aqeuous) to a final concentration of 50 μM. The ACP solution was dialyzed against Tris-HCl buffer (50 mM, pH 8.0, aqeuous) initially containing 8 M urea, and the urea concentration was incrementally reduced by 1 M every 12 h until the buffer was devoid of denaturant. ACP was recovered by anion-exchange chromatography on a MonoQ column (Amersham Pharmacia) eluting with a linear gradient of 0−1 M NaCl over 8 column volumes, desalted into MilliQ water, and lyophilized.

(B) Rapid Dilution. Lyophilized synthetic apo-ACP (3 mg) was resuspended in aqueous guanidine HCl (50 μL, 6 M) and incubated at 30 °C for 1 h. The resultant denatured protein solution was pipetted in a single step into a potassium phosphate buffer (10 mL, 100 mM, pH 7.3) while stirring rapidly, and 80% of the protein was recovered by anion-exchange chromatography and desalting as described above.

GroEL/ES. Initially to test the chaperone activity of GroEL/ES, a sample of recombinant C17S act apo-ACP (C17S apo-ACP) (3 mg) was denatured by heating at 100 °C for 16 h on a Grant BT3 heat block. Disruption of the structure was confirmed by CD and 1D ^{1}H NMR. The ACP was then lyophilized, and 1 mg was resuspended in aqueous guanidine hydrochloride (50 μL, 6 M) and incubated at 30 °C for 1 h. The resultant denatured protein solution was pipetted in a single step into CHAPS buffer (10 mL, 50 mM Tris-HCl, pH 7.3, 50 mM KCl, 20 mM DTT, 2 mM $MgCl_2$) containing GroEL (10 μM) while stirring rapidly. Folding was induced by the addition of GroES (20 μM) and ATP (5mM) and incubated for 2 h at 30 °C. Synthetic apo-ACP (3 mg) was refolded using the GroEL/ES chaperonin system as described above for heat-denatured C17S apo-ACP (*29*). ACP was then purified using gel filtration.

Phosphopantetheinylation of Apo-ACP. Phosphopantetheinylation of apo-ACP to yield holo-ACP was achieved by incubation of purified apo-ACP (100 μM) with purified *S. coelicolor* His_6-ACPS (1 μM) and coenzyme A (1 mM) in the presence of DTT (5 mM), $MgCl_2$ (10 mM), and Tris-HCl (50 mM), pH 8.8 (*28*, *34*). Reactions were incubated at 30 °C for 16 h, and apo to holo conversion was confirmed by ESMS. ACP was then purified using gel filtration.

Self-Malonylation Assay. Holo-ACP (50 μM) was incubated with malonyl-CoA (50 μM) at 30 °C in a total volume of 100 μL in the presence of potassium phosphate buffer (100 mM, pH 7.3) and glycerol [10% (v/v)] (*19*, *21*). Reactions were quenched after 30 min by addition of C4 resin in methanol and prepared for ESMS.

Minimal Polyketide Synthase Assays. In vitro minimal PKS assays were performed as reported previously (*21*). *S. coelicolor* His_6-KSα:KSβ (1 μM) was incubated (10 min, 30 °C) with monomerized holo-ACP (50 μM) in potassium phosphate buffer (50 mM, pH 7.5, 2 mM EDTA) to allow the formation of the active complex. Polyketide production was initiated by the addition of malonyl-CoA (1 mM). Assays were incubated for 2 h at 30 °C with shaking. Assays were quenched by addition of potassium phosphate (100 mg) and extracted into diethyl ether (300 μL) three times. The samples were allowed to evaporate under a stream of nitrogen at room temperature, and the sample was then resuspended in acetonitrile (50 μL). The resuspended sample was analyzed by reverse-phase HPLC using a Luna C18(2) 250 × 4.6 mm reverse-phase column (Phenomenex) on a Gilson Instruments HPLC. The column was equilibrated for 5 min after injection of the sample with water with 5% acetonitrile and 0.05% (v/v) trifluoroacetic acid (TFA) at a flow rate of 1 mL/min. The concentration of acetonitrile was then linearly increased to 75% over 30 min. Compounds were detected by monitoring the absorbance at 280 nm. SEK4 was found to elute at approximately 19.5 min followed by SEK4b at 20.5 min.

RESULTS

Synthesis of WT Apo-ACP. Synthetic apo-ACP was produced using solid-phase peptide synthesis and purified by HPLC to yield a total of 18.1 mg of protein (Figure 1A). The mass was confirmed by mass spectrometry and compared to the wild-type protein lacked the N-terminal methionine as expected (observed 9116.3 Da; calculated 9115.9 Da) (Figure 1C). C17S apo-ACP was also purified to homogeneity as described previously (*26*). Purity was again confirmed by ESMS and HPLC. Both proteins eluted with the same retention time and as a single peak by HPLC (Figure 1A). SDS−PAGE showed bands at 3.5 kDa characteristic of act ACP (*26*) (Figure 1B). CD spectra in the far-UV region were routinely recorded for all ACPs in this study (Figure 2). The CD spectrum of folded recombinant C17S apo-ACP is clearly characterized by two intense

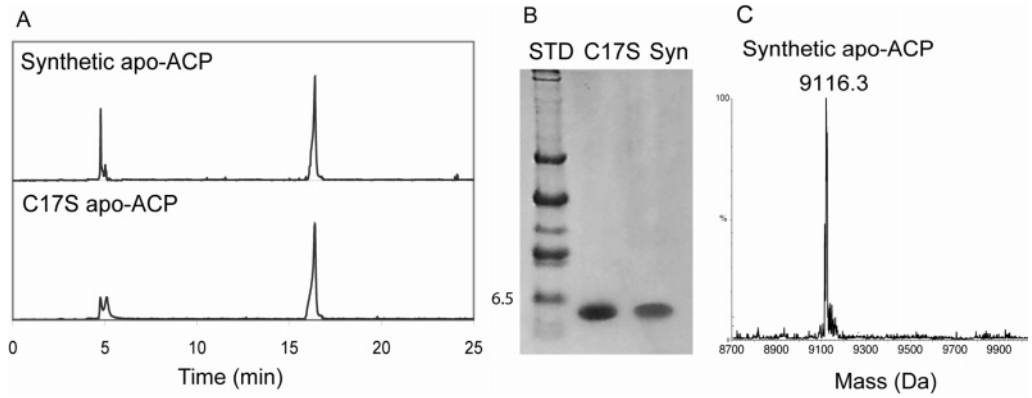

FIGURE 1: HPLC, SDS−PAGE, and ESMS spectra of synthetic apo-ACP and C17S apo-ACP. (A) Reverse-phase HPLC trace of synthetic ACP (top trace) and C17S (bottom trace). Both proteins elute after approximately 16 min. (B) SDS−PAGE analysis of the recombinantly expressed and purified C17S apo-ACP used throughout and the synthetic (syn) apo-ACP. (C) Transformed ESMS spectra of the synthetic apo-ACP (observed mass 9116.3 Da; calculated mass 9116.48 Da).

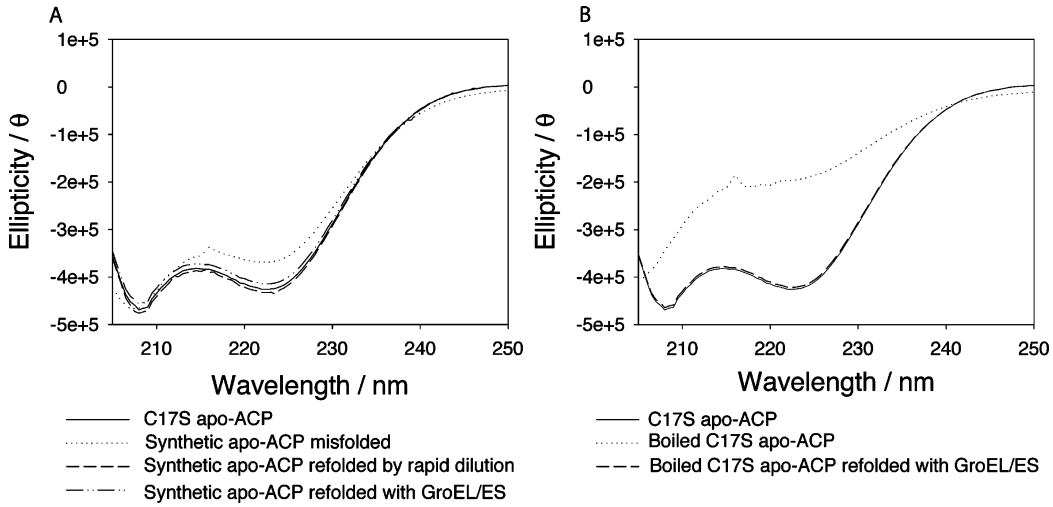

FIGURE 2: (A) Comparison of CD spectra of C17S apo-ACP with unfolded synthetic apo-ACP, synthetic apo-ACP refolded by rapid dilution, and synthetic apo-ACP refolded with GroEL/ES. (B) Comparison of CD spectra of C17S apo-ACP with heat-denatured C17S apo-ACP and heat-denatured C17S apo-ACP refolded with GroEL/ES.

negative bands at 208 and 223 nm. This absorption is indicative of predominantly α-helical secondary structure. The CD spectrum of the misfolded synthetic apo-ACP (Figure 2A) shows the same negative absorption bands at 207 nm and a slightly weaker absorption at 223 nm, suggesting that the secondary structure content of this ACP approached that of folded C17S apo-ACP.

The synthetic apo-ACP was checked for correct folding by comparison of its 1D ^{1}H NMR spectrum with that of C17S apo-ACP (*32*, *33*). Figure 3A shows the 1D ^{1}H spectrum of C17S apo-ACP recorded at 600 MHz and 25 °C. The spectrum is of high quality and shows the characteristic ^{1}H envelope of a folded protein. Several resolved signals indicate correct folding of ACP. These include the upfield-shifted methyl signal of Leu45 δMe at 0.22 ppm and the three downfield signals in the amide region from Glu36, Leu5, and Asp69 (indicated in Figure 3A). The 1D ^{1}H spectrum of misfolded synthetic apo-ACP is shown in Figure 3B recorded under identical temperature and buffer conditions. This spectrum shows broad and poorly dispersed peaks not characteristic of folded act ACP. The profile also clearly lacks the upfield-shifted and downfield-shifted peaks that are easily resolved in the C17S apo-ACP spectrum.

Initial Assays of Recombinant C17S Actinorhodin Apo-ACP and Synthetic Actinorhodin Apo-ACP. As we have previously shown, C17S apo-ACP is a substrate for the *S. coelicolor* holo-ACP synthase (ACPS) (*34*) converting apo- to holo-ACP. This conversion gave 100% holo in 120 min characterized by ESMS (observed 9439 Da; calculated 9441 Da) (*28*) (Figure 4A). When incubated with malonyl-CoA, malonate was transferred to the phosphopantetheine thiol, achieving 70% completion in 30 min (ESMS characterization: observed 9527 Da; calculated 9527 Da) (Figure 4C). Finally, recombinant C17S act holo-ACP (C17S holo-ACP) was incorporated into the in vitro minimal assay, and SEK4 and SEK4b production was determined by HPLC separation of the products as described previously (*21*) (Figure 4B). The misfolded synthetic apo-ACP protein showed no post-translational modification using *S. coelicolor* ACPS and, therefore, could not be a substrate for either self-malonylation or the minimal assay.

Refolding of Synthetic Actinorhodin Apo-ACP. Given the initial ^{1}H NMR and CD spectra indicated that synthetic apo-ACP was not folded correctly, the lack of function of the ACP was not unexpected. We proceeded therefore to refold synthetic apo-ACP using a variety of techniques. Initially, denaturation of synthetic apo-ACP with urea followed by stepwise dialysis yielded protein that was not modified to the holo form by *S. coelicolor* ACPS and assayed identically to the original misfolded synthetic protein. Secondly, rapid

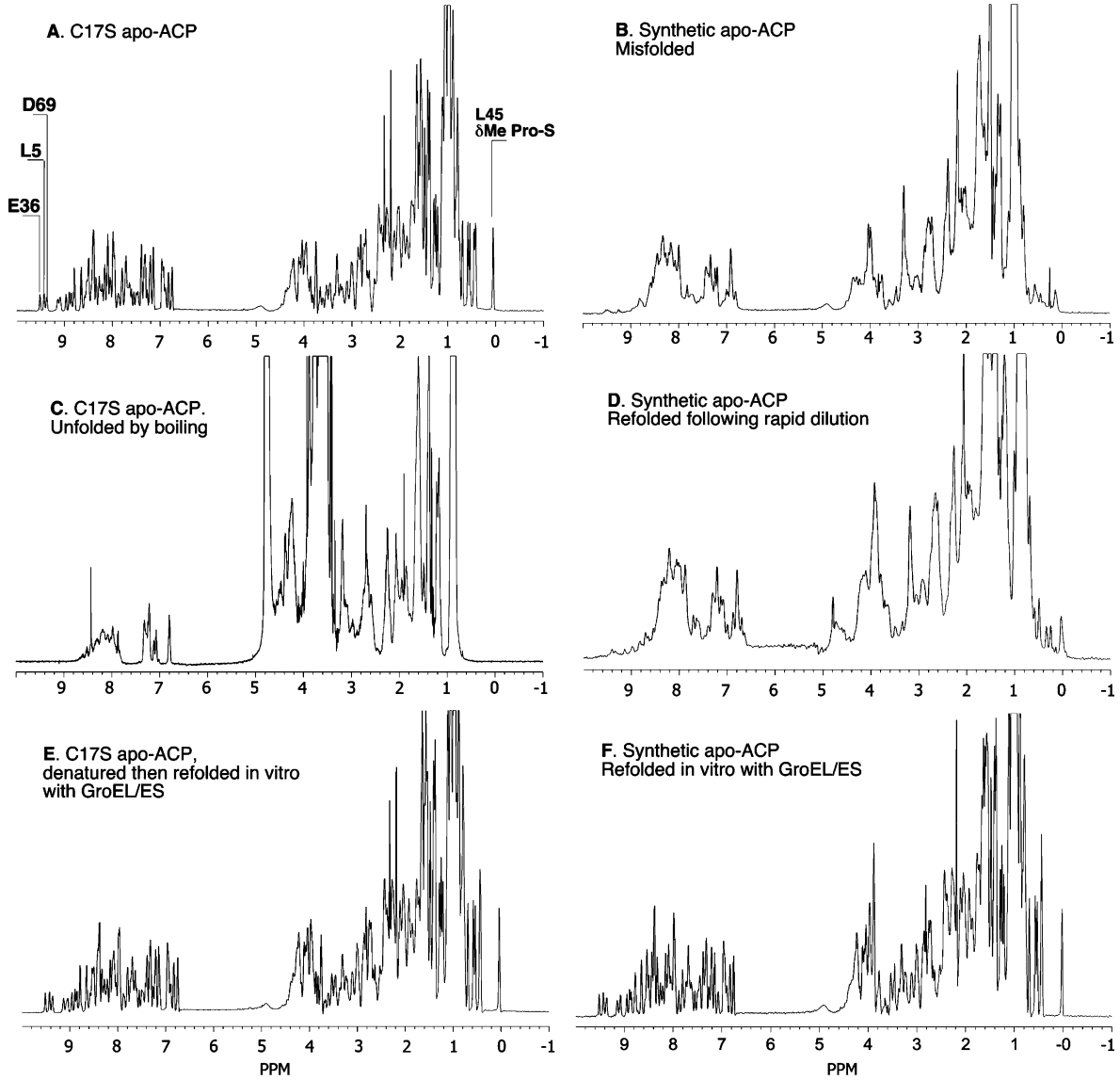

FIGURE 3: 1D ^{1}H NMR spectra collected in 20 mM K_2HPO_4 and 1 mM NaN_3, pH 5.5, with 5% D_2O and at 25 °C. (A) Spectrum of C17S apo-ACP expressed and purified as reported previously (*26*). The characteristic shifts of three amide protons (Glu36, Leu5, and Asp69) are marked with arrows in the downfield region of the spectrum. The characteristic upfield-shifted methyl group of Leu45 is also marked. (B) Spectrum of synthetic apo-ACP following HPLC purification and freeze-drying with no refolding steps. (C) C17S apo-ACP denatured by boiling for 16 h. (D) A misfolded form of synthetic apo-ACP characterized after attempted refolding with rapid dilution. (E) C17S apo-ACP from (C) after refolding with GroEL/ES preparations. (F) Synthetic apo-ACP from (B) after refolding with GroEL/ES preparations.

dilution methods were applied to the protein (*35*). Starting with 2.2 mg of protein, 1.6 mg of protein were recovered and once again assayed using the methods outlined above. The CD spectrum of this protein is shown in Figure 2A and shows a curve identical to that of recombinant C17S act apo-ACP, suggesting that both proteins have a similar secondary structure content. The ^{1}H NMR spectrum (Figure 3D) indicated, however, a profile that was in fact only slightly modified from the original unfolded synthetic spectrum and was not comparable to the well-resolved 1D spectrum of C17S apo-ACP. This suggested that the synthetic apo-ACP was partially but not correctly folded. When assayed, however, this synthetic apo-ACP sample was now a substrate for the ACPS, being converted to 100% of the holo form in 16 h (observed 9456.2 Da; calculated 9455 Da) (data not shown). However, malonate was not transferred from malonyl-CoA to the phosphopantetheine thiol, nor did the ACP support polyketide production in the minimal assay. We also attempted to acylate this ACP using *S. coelicolor* MCAT [purified as described previously (*21*)] and malonyl-CoA. Again, we did not observe any malonylation of the synthetic act holo-ACP (synthetic holo-ACP). This was compared to a *S. coelicolor* FAS ACP control which was converted to 80−90% malonyl-ACP by MCAT over the full 30 min time course. Finally, incorporation of the partially folded synthetic holo-ACP in the presence and absence of *S. coelicolor* MCAT into the minimal system failed to support polyketide production.

The use of GroEL/ES to refold recombinant proteins in vitro has been reported (*29*). Heat-denatured C17S apo-ACP and synthetic apo-ACP were refolded over 2 h with a stoichiometric amount of purified GroEL/ES at 37 °C in the presence of an excess of ATP. Refolded ACP was separated from GroEL/ES by size exclusion chromatography on a Superdex 75 column (GE Healthcare). When examined by CD, the refolded synthetic apo-ACP spectrum showed a

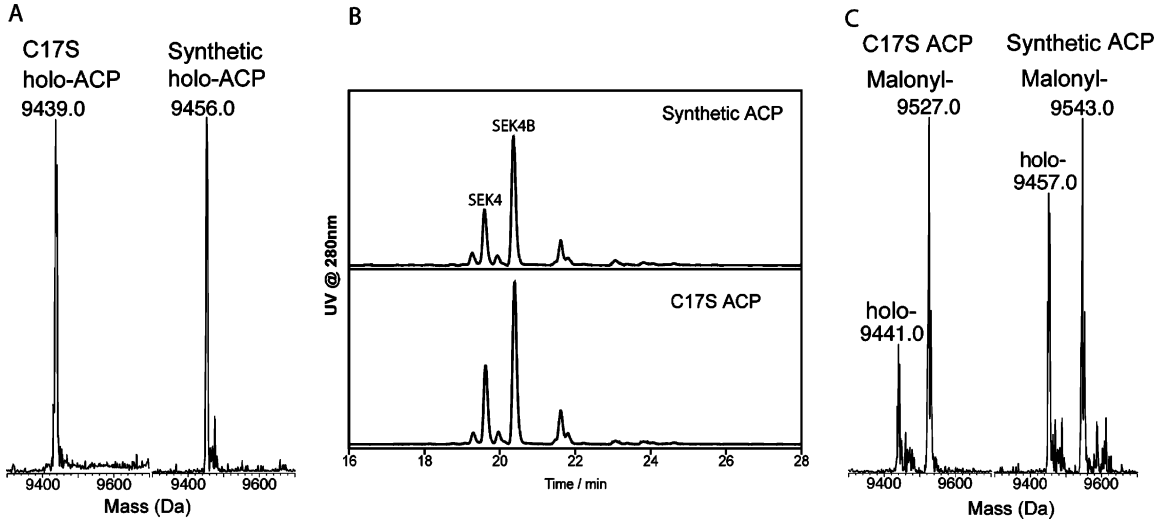

FIGURE 4: (A) Transformed ESMS spectra of phosphopantetheinylated C17S apo-ACP and GroEL/ES refolded synthetic apo-ACP. (B) Typical HPLC trace of minimal polyketide synthase assay comparing the ability of the GroEL/ES refolded synthetic apo-ACP and C17S apo-ACP to support SEK4 and SEK4b production. (C) Transformed ESMS spectra of a typical self-malonylation assay after 30 min comparing the self-malonylation ability of C17S holo-ACP with the GroEL/ES refolded synthetic holo-ACP.

superimposable trace with that of C17S apo-ACP (Figure 2A). Similarly, the ^{1}H NMR spectrum showed good peak dispersion and narrow line widths and was indistinguishable from C17S apo-ACP (Figure 3F). A 2D ^{1}H NOESY spectrum was also acquired (data not shown) that revealed the presence of the expected NOEs for the correctly refolded form of C17S apo-ACP. Heat-denatured C17S apo-ACP (CD spectrum, Figure 2B; ^{1}H NMR, Figure 3C) was also shown to be inactive in all the assays but was successfully refolded using the GroEL/ES cochaperone system (Figure 3E). Heat-denatured and refolded recombinant ACP was converted to 100% holo-ACP by ACPS and malonylated in the presence of malonyl-CoA to 70% in 30 min (data not shown). Finally, this refolded recombinant protein supported polyketide biosynthesis in the minimal PKS without the need for MCAT. Therefore, the chaperone system appears to be the method of choice to successfully refold either heat-denatured or synthetic apo-ACP.

Self-Malonylation of Synthetic Actinorhodin Apo-ACP. Refolded synthetic apo-ACP was modified to holo-ACP using *S. coelicolor* ACPS, which gave 100% conversion in 30 min (Figure 4A). Synthetic holo-ACP was then incubated with malonyl-CoA, and self-malonylation was observed, reaching 60% in 30 min (vs 74% for recombinant holo-ACP) (Figure 4C). Incorporation of synthetic holo-ACP into the minimal system produced the polyketides SEK4 and SEK4b to 83% of the level of C17S holo-ACP (Figure 4B).

As further controls *S. coelicolor* ACPS successfully modified *S. coelicolor* FAS apo-ACP to the holo form, but this mixture did not initiate malonylation upon addition of malonyl-CoA. Further, the same batch of purified *S. coelicolor* FAS ACP was confirmed to be a substrate for MCAT when assayed separately with *S. coelicolor* MCAT, confirming that the FAS ACP was folded correctly. Finally, an incubation of GroEL or GroES, *S. coelicolor* FAS holo-ACP, and malonyl-CoA gave no modification of the FAS ACP. These controls prove that there was no contamination of either ACPS or GroEL/ES.

DISCUSSION

The in vitro self-malonylation of type II PKS ACPs has been a subject of debate since it was first reported in 1998 (*19*). The observation that these ACPs self-acylate using a variety of CoA or *N*-acetylcysteamine thiol esters has been reported by several groups (*19*−*23*). The phenomenon of self-malonylation, however, has been refuted by one group (*36*), retracted subsequently by another (*24*), and in both cases attributed to minor levels of MCAT contamination in the ACP preparations used and not specifically to the catalytic ability of the ACP. Controls used to demonstrate self-malonylation in the original studies (*19*) used a serine-specific inhibitor, PMSF, at sufficient concentrations to inhibit MCAT (*37*). The effectiveness, however, of PMSF as an MCAT inhibitor in the self-malonylation assays was challenged by Khosla and co-workers, who reported that the removal of the active site S97 of *S. coelicolor* MCAT did not inhibit the functioning of a minimal PKS in vitro. As a result they proposed that S97 was not essential for catalysis by this enzyme, and thus a second active site catalytically competent nucleophile must be present (*36*). In support of this they subsequently reported that both S97A and H96A mutants were labeled upon incubation with [^{14}C]malonyl-CoA and were able to transfer malonate from malonyl-CoA to the heterologous fren ACP. As such, they proposed that H96 was a second "surrogate" nucleophile for malonyl transfer (*38*). Subsequent work, however, did not support this idea and indicated that S97 was indeed the sole active site nucleophile involved in malonyl transfer (*27*). This was confirmed when the three-dimensional structure was solved and shown to have an active site architecture common to all known acyl transferase enzymes (*39*). There is therefore no evidence to suggest that PMSF does not inhibit MCAT. The retraction by Reynolds and co-workers was based on the heterologous expression of tcm ACP in insect cells, a host that has no type II FAS or discrete MCAT. When expressed and purified, the tcm ACP did not undergo self-malonylation but was a substrate for *E. coli* MCAT (*24*). It was impossible for this system to have an MCAT contamination, therefore apparently ruling out self-malonylation. Notably, neither of

the above studies addressed the observation that type II FAS PKSs expressed and purified using exactly the same protocols as their type II PKS counterparts do not undergo self-acylation (*19*). In addition, none of these studies, which appear to present strong evidence refuting the observation of self-malonylation, have provided any structural characterization of the ACP component that is being studied. In the absence of any structural characterization we believe that no firm conclusions on function can be made. We have therefore applied our knowledge of PKS ACPs, combined with NMR and chemical synthesis, to give assays that are intrinsically free of MCAT with fully structurally characterized ACP components. In this study we have now shown conclusively that the observed self-acylation of PKS ACPs is an intrinsic property of these proteins which must therefore be considered to be enzymes in their own right.

We have taken the obvious approach of using synthetic apo-ACP synthesized by stepwise solid-phase peptide chemistry (*25*). Solid-phase peptide synthesis has the advantage of yielding protein that is intrinsically free from contamination. The synthetic apo-ACP was misfolded when examined by CD and ^{1}H NMR and was inactive in all modification reactions and assays. We initially explored the use of two chemical refolding methods, stepwise dilution and rapid dilution. Stepwise dilution yielded ACP that gave identical ^{1}H NMR and CD spectra to that of the starting material and was not modified to the holo form by ACPS. Rapid dilution gave a CD spectrum that is essentially identical to that of C17S apo-ACP but showed only a minor modification of the ^{1}H NMR spectrum. CD is a lower resolution technique and on its own would provide a misleading guide to the fidelity of refolding. This partially folded iso form was a substrate for the *S. coelicolor* ACPS, giving 80% modification to the holo form. This protein did not, however, self-malonylate when incubated with malonyl-CoA, nor was it active as a substrate in the MCAT assay. The decoupling of phosphopantetheinylation and malonylation is a surprising result but may point to the requirement for different recognition motifs on the ACP for ACPS and MCAT enzymes. It is known from previous mutational and structural studies of the interaction of *E. coli* FAS ACP with ACPS and MCAT that the necessary protein−protein interactions are sensitive to amino acid and structural changes in the helix 2−loop−helix 3 region (*40*, *41*). Interestingly, the tcm ACP from baculovirus expression clearly has a better defined structure than the ACP formed following rapid dilution as it was a substrate for ACPS but was also a substrate for MCAT (*24*). However, in the absence of structural information the fact that self-malonylation did not occur could indicate that the protein had not attained the correctly folded state.

The correct folding of synthetic apo-ACP was finally achieved by using a GroEL/ES cochaperone system. This form was again confirmed by CD, but in this case high-resolution 1D and 2D ^{1}H NMR gave identical spectra to that of the wild-type protein. The correctly folded synthetic apo-ACP underwent phosphopantetheinylation with ACPS as well as self-malonylation in the presence of malonyl-CoA (with no MCAT present). A similar process yielded refolded C17S apo-ACP from a heat-denatured form, and this preparation was a substrate for ACPS and also undergoes self-malonylation. Both proteins were active in the minimal assay without the requirement for *S. coelicolor* MCAT. In the course of all the experiments described we have used rigorous controls to ensure that the use of any additional enzymes could not introduce a contamination. Previously, Reynolds also used ACPS to convert the apo-ACP expressed and purified from baculovirus to the holo form without the introduction of any contaminating MCAT (*24*). In our experiments, the natural substrate for MCAT, *S. coelicolor* FAS ACP, was recombinantly expressed in *E. coli*, purified, and modified to the holo form using purified *S. coelicolor* ACPS. No malonylation of the FAS ACP negative control was observed, showing that the purified *S. coelicolor* ACPS could not introduce MCAT contamination. Further, the *S. coelicolor* FAS ACP did malonylate when assayed with MCAT and malonyl-CoA, proving the structural integrity of the FAS ACP used in this control.

Despite much evidence to the contrary, it continues to be frequently stated in current literature that the minimal PKS consists of four components: KS, CLF, ACP, and MCAT (*42*, *43*). It needs to be emphasized that all of these studies relate to the in vitro situation and it is not possible to be categorical about the true in vivo situation. What is notable is that of all known FAS and PKS systems which utilize ACPs, only the type II PKSs lack a dedicated MCAT. The work described above and previous studies from our laboratory demonstrate that type II PKSs do indeed require a malonyl transferase activity but that this activity can be satisfied, in vitro at least, by the inherent malonyl transferase activity of the endogenous ACP component. Kinetic studies have clearly shown that malonylation of ACP is the rate-limiting step and the rates of polyketide (SEK4 + SEK4b) production reported for in vitro PKS assays are effectively the rate of malonylation of ACP (*21*). This observation may or may not provide an explanation for the remarkable lack of dedicated MCAT genes in these clusters, but they do demonstrate beyond reasonable doubt that our preparations of ACPs are free from contamination with *E. coli* MCAT and that the in vitro minimal act PKS requires only three components, KS, CLF, and ACP.

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