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Acylation of *Streptomyces* type II polyketide synthase acyl carrier proteins

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Abstract Acyl derivatives of type II PKS ACPs are required for in vitro studies of polyketide biosynthesis. The presence of an exposed cysteine residue prevented specific chemical acylation of the phosphopantetheine thiol of the actinorhodin PKS *holo* ACP. Acylation studies were further complicated by intramolecular disulphide formation between cysteine 17 and the phosphopantetheine. The presence of this intramolecular disulphide was confirmed by tryptic digestion of the ACP followed by ESMS analysis of the fragments. An act Cys17Ser ACP was engineered by site-directed mutagenesis. *S*-Acyl adducts of act C17S, oxytetracycline and griseusin *holo* ACPs were rapidly formed by reaction with hexanoyl, 5-ketohexanoyl and protected acetoacetyl imidazolides. Comparisons with type II FAS ACPs were made.

Key words: Acyl carrier protein; Acylation; Polyketide synthase; Fatty acid synthase

1. Introduction

Polyketides are a heterogeneous group of secondary metabolic natural products possessing wide ranging pharmacological activities [1]. Like the structurally and mechanistically related fatty acid synthases (FASs) [2], polyketide synthases (PKSs) catalyse repeated decarboxylative condensations between simple acyl thioesters. Each cycle results in the formation of a β-keto group and by selectively controlling the extent of reduction of these groups PKSs generate great structural variability [3].

PKSs of bacteria can be classified into two groups. Type I synthases are large multifunctional proteins which catalyse the biosynthesis of highly reduced polyketides such as the macrolide erythromycin [4]. Distinct catalytic sites are organised linearly in such a way that the polyketide may be synthesised using the enzymes in a non-repetitive manner. Type II synthases catalyse the biosynthesis of aromatic polyketides in streptomycetes. They are multienzyme complexes formed from up to eight individual enzyme activities that function iteratively. The β-keto groups of the intermediates formed by these type II complexes remain largely unmodified allowing the highly reactive polyketide backbone to undergo a series of enzyme catalysed, regiospecific cyclisations [5]. Understanding the factors which control polyketide production, particularly in the type II enzyme systems, is a major challenge in current biosynthetic studies.

Several laboratories have recently generated a number of hybrid polyketide synthases which have facilitated the analysis of both modular [6,7], as well as aromatic PKSs [8]. Using such genetics based combinatorial techniques a number of novel compounds have been produced, the analysis of which has enabled the enzymes responsible for controlling the specificity of individual reactions within the overall catalytic cycle to be identified. Many of these deductions are largely inferential, however, and are complicated by the fact that the genetic experiments often fail to produce an isolable product.

Direct studies on isolated PKSs in vitro should provide invaluable information, although to date relatively few intact PKSs have been purified to homogeneity and examined. Active cell-free preparations of both type I [9,10] and type II PKS [11,12] enzymes have been demonstrated. A fully active cell-free type II PKS, the tetracenomycin (tcm) producing PKS from *Streptomyces glaucescens*, was identified in 1993 [11], while active preparations of both the complete actinorhodin (*act*) PKS from *S. coelicolor*, as well as a minimal system have been obtained [12]. The fact that the type II PKSs exist as an assembly of discrete proteins may make it impossible to purify an intact PKS. In the light of this, constituent proteins are being isolated individually in an attempt to establish their physical and biochemical characteristics. This should allow the reconstitution of combinations of these isolated proteins in vitro, complementing the mix and match genetic studies.

To date the best studied PKS protein is the acyl carrier protein (ACP), which has been isolated (predominantly in the inactive *apo* form) from a number of type II *Streptomyces* systems [13,14]. Both the secondary structure [15] and three dimensional solution structure [16] of the actinorhodin (act) ACP have been determined by two dimensional (2D) NMR techniques. The tertiary fold of *apo* act ACP shows a strong structural homology with *Escherichia coli* FAS ACP for which a solution structure is also available [17,18]. Both the PKS and FAS ACPs consist of four helices which form a hydrophobic cleft capable of accommodating both the 4′-phosphopantetheine prosthetic group as well as an acyl chain. In the case of the PKS protein, however, a number of hydrophilic groups have been identified, buried within the core of the protein which may play a role in the stabilisation of the growing polyketide chain [16]. A further difference between the PKS and FAS ACPs relates to the dynamic properties of the two structures. The *E. coli* FAS ACP is thought to exist

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Abbreviations: ACP, acyl carrier protein; act, actinorhodin; ESMS, electrospray mass spectrometry; FAS, fatty acid synthase; gris, griseusin; NPDS, *p*-nitrophenyl disulphide; NPS, nitrophenyl sulphide; PKS, polyketide synthase; otc, oxytetracycline

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in two or more distinct conformations [19,20] while only a single conformation has been observed for the PKS ACP [16]. The significance of these findings is at present unknown, but may reflect the relative capacities of these proteins to change conformations whilst accommodating their respective acyl chains.

The interactions between the acyl chain and *E. coli* FAS ACP have been extensively studied. The acyl binding cleft encloses the first six to eight carbon atoms of the acyl chain [21–23] reducing the hydrodynamic radius of the protein, and rendering it more stable to expansion at alkaline pH. This suggests that some interactions may exist between the acyl chain and the amino acids within the binding cleft. Despite these initial physical observations, two-dimensional NMR studies of acyl ACP showed that the structural changes which occur following acylation with fatty acids of eight carbon atoms or less are small [24,25].

As part of our on-going study of the polyketide ACPs we have set out to specifically acylate purified type II PKS ACPs with both fully reduced acyl chains, as well as polyketide assembly intermediates, establishing the efficiency of both chemical and enzymic methods. This paper describes the acylation of *act* and other polyketide *holo* ACPs and their detailed characterisation.

2. Materials and methods

2.1. ACP derivatisation and ESMS analysis

The *apo* and *holo* forms of griseusin, oxytetracycline, actinorhodin and an actinorhodin cysteine 17 serine mutant (described in this paper) PKS ACPs were expressed and isolated as previously described using standard techniques [14,26]. For the derivatisation experiments an aqueous solution of ACP (25 μl at a concentration of 4 mg/ml), adjusted for pH (range 5–9) by the addition of either dilute HCl or ammonia, was treated with a molar equivalent of *p*-nitrophenyl disulphide (NPDS) in acetonitrile (5 μl). This reaction was quenched by the addition of a 25% formic acid solution (5 μl) after a number of timed intervals up to 1 h. Mass spectra were measured on a VG Quattro spectrometer equipped with an electrospray source. Each sample (20 μl) was directly injected into the electrospray source via a Rheodyne loop injector at a solvent (water:acetonitrile 50:50 v/v, 1% formic acid) flow rate of 20 μl/min. The mass spectrometer was routinely scanned over the *m/z* range 800–1600 and this mass range was calibrated using a solution of horse heart myoglobin (20 pmol injections; $M_r = 16951.48$ Da). Wild type *holo* ACP solutions containing a 5-fold excess of DTT were incubated at 37°C for 30 min before desalting (FPLC G25 fast desalt column, Pharmacia) using water at the pH at which the ESMS experiments were performed. Analyses were carried out immediately. Imidazolide derivatives were prepared using the methods described by Cronan and Klages [27] and ACP derivatisations carried out as with the NPDS experiments.

2.2. ACP mutation procedure

*Act*I-ORF3 (ACP) was cloned as an *Xba*I fragment from pIJ 5467 into the *Xba*I site of pUC118. One clone, in which the *act*I-ORF3 ran in the *Eco*RI to *Hin*dIII direction, was designated pRJCI117d. Two synthetic oligo nucleotides were constructed (mutation site underlined): 5′-GACGGACGGGACGGACGTC-3′ and 5′-TCACCGGC-GTCTCCACGA-3′. 1.0 nmol of each was treated with 5 units of T4 polynucleotide kinase in a 10 μl reaction for 45 min at 37°C, the reactions were then heat treated at 90°C for 2 min. The oligonucleotides (0.1 nmol of each) were used to prime a standard polymerase chain reaction (PCR), containing pRJCI117d (0.2 μg), 6% glycerol and VENT DNA polymerase (1 unit). Temperature was cycled as follows: 96°C, 60 s, then 60°C, 45 s, then 72°C, 150 s, repeated 25 times, then 4°C. The resultant synthetic linear plasmid DNA was precipitated and resuspended in 100 μL H_2O. 20 μl of this solution was digested with *Dpn*I at 37°C for 2 h. The DNA was precipitated and resuspended in 50 μl H_2O. 8 μl of this DNA was ligated in a 10 μl reaction with T4 DNA ligase (0.5 units) at 12°C for 2 h. DNA was precipitated and resuspended in 10 μL H_2O. 4 μl was electroporated into *E. coli* JM101. The resultant cell suspension was spread for single colonies on L agar containing 100 μg/ml carbenicillin.

Twelve single colonies were picked for sequence analysis. Single stranded DNA was generated using helper phage M13KO7, and purified prior to sequencing using the method of Boyle and Lew [28]. Sequencing was carried out by hand. The required mutation was apparent in six out of 11 clones: no other mutations were observed. One mutant clone was digested with *Nde*I and *Bam*HI and the resultant fragment subcloned into the *Nde*I and *Bam*HI sites of the expression vector pT7-7. Expression of the resultant strain (pIJ2366) was performed by standard procedures and derivatisation experiments carried out exactly as for the non-mutated act ACP.

2.3. Tryptic digest of PKS ACPs

Wild type *apo* act ACP and *holo* act ACP (100 μM) were digested with 2% (w/w) trypsin (sequencing grade, Sigma) in 100 mM NH_4HCO_3 (pH8.0) for 4 h at 37°C. 50 μl of digested protein was injected onto a C_{18} reverse phase HPLC column (0.4×240 mm, 300 Å, Rainin) equilibrated in milli Q water containing 0.1% TFA. The peptide fragments were eluted with a 0–100% acetonitrile+0.085% TFA gradient (over 110 min) at 0.5 ml/min monitoring absorption at 214 nm. Fractions containing peptide fragments were lyophilised, resuspended in water:acetonitrile (50/50 v/v) containing 1% formic acid and analysed by ESMS (20 μl injections) as described previously.

2.4. Acyl ACP synthetase assay

Holo ACP (1 mg/ml, 15 μl) was incubated with acyl ACP synthetase (Sigma, 2 mU) at 37°C for 1 h in Tris-HCl (100 mM, pH 8.0) containing DTT (2 mM), ATP (5 mM), LiCl (0.4 M), Triton X-100 (2%), palmitic acid (3 mM) and ^{14}C-labelled palmitic acid (2 μl stock solution, specific activity 824 mCi/mmol), in a final volume of 50 μl. An aliquot (25 μl) of the incubation mixture was spotted onto the end of a 10 cm strip of Whatman 3MM filter paper and excess, unbound radiolabel eluted away from the radiolabelled ACP using a chloro-

Table 1
Masses of chemically acylated derivatives of *holo* gris and otc ACPs

Acyl group	*Holo* gris ACP[a]		*Holo* otc ACP[b]	
	Expected mass (Da)	Observed mass (Da)	Expected mass (Da)	Observed mass (Da)
–	9885	9886.8 ± 1.0	10254	10257.8 ± 1.5
	10016	10015.9 ± 2.9		
Hexanoyl	9982	9982 ± 2.0	10352	10352.2 ± 0.6
	10113	10116.0 ± 1.3	10450	10451.1 ± 2.9
5-Ketohexanoyl	9996	9996.7 ± 1.3	10366	10367.8 ± 1.7
	10127	10127.8 ± 2.9	10478	10481.4 ± 1.2
Protected acetoacetyl	10013	10013.9 ± 3.9	10383	10385.6 ± 1.1
	10144	10144.0 ± 0.7	10512	10515.4 ± 1.3

[a]Gris ACP exists in two forms as a result of partial endoproteolytic cleavage of the N-terminal methionine and so two peak series were obtained in each ESMS spectrum.
[b]Otc ACP has an exposed cysteine residue. Formation of mono and bis adducts by acylation of both the 4′-phosphopantetheine and the cysteine (Cys-16) thiols was observed.

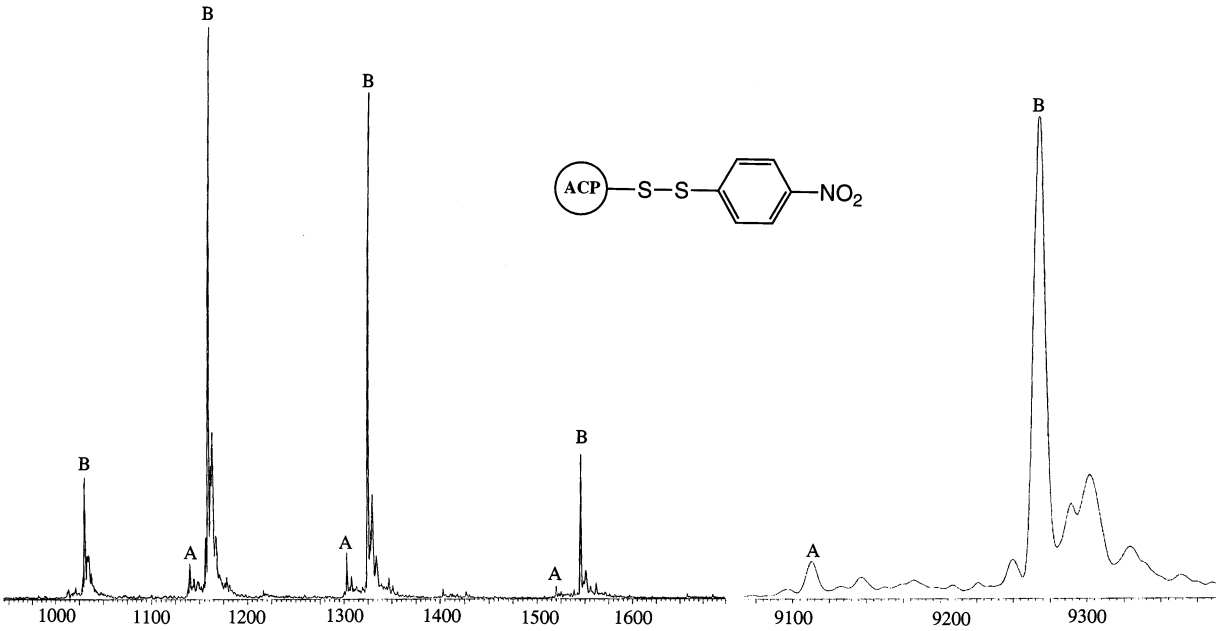

Fig. 1. ESMS spectrum of the reaction of NPDS with the *apo* ACP. A: *apo* act ACP, expected $M_r = 9117$, observed = 9116.10 ± 1.0; B: NPS-*apo* act ACP: expected $M_r = 9270$, observed = 9269 ± 0.7.

form:methanol:acetic acid (3:6:1 v/v) mixture. After the filter papers had dried thoroughly the washing procedure was repeated and the papers dried again. The filter papers were then cut into 2.5 cm sections, placed in scintillation fluid, and counted for radioactivity. Commercially available *E. coli* ACP (Sigma) was used as a control in these experiments.

3. Results and discussion

Our knowledge of the factors controlling the type II polyketide synthase complex has progressed rapidly over the past few years with the demonstration that novel aromatic compounds can be genetically engineered through the combinatorial manipulation of PKS subunits [7,8]. Attempts to repeat these combinatorial studies in vitro in order to provide an alternative route for the controlled synthesis of novel polyketides have been hampered by the intrinsic nature of bacterial type II systems. Purification or reconstitution of the complete PKS complex has not been reported, although active cell-free systems have been obtained [11,12]. At least one individual *Streptomyces* type II PKS component, the acyl carrier protein has been successfully expressed in *E. coli*, purified [13,14], obtained predominantly in an active *holo* form [26] and a 3 dimensional solution structure solved [16]. In this paper we have further characterised the actinorhodin PKS ACP and demonstrated that acylation of this and other type II PKS ACPs can be accomplished using chemical techniques previously used to derivatise FAS ACPs [27].

The thiol specific reagent NPDS reacted rapidly with the *apo* form of act ACP. ESMS analysis showed greater than 90% addition of nitrophenyl sulphide (NPS) to this ACP species occurred after 5 min incubation at pH 6–8 (Fig. 1). At pH 5, 60–65% was derivatised after 5 min, rising to 90% after 1 h, suggesting a slight pH effect on this reaction. These experiments show that *apo* act ACP contains an exposed thiol group. In the absence of the phosphopantetheine co-factor the only candidate is cysteine 17. This amino acid has been previously implicated in the rapid dimerisation of both actinorhodin and oxytetracycline (otc) *apo* ACPs [14]. Surprisingly, initial ESMS analyses of *holo* act ACP after incubation with NPDS showed no addition of NPS to the protein if the reaction was carried out at pH 7 or higher. A mass of 9455.5 ± 1.5 was obtained, corresponding to the expected mass of the *holo* protein. In this case neither the cysteine thiol nor the 4′-phosphopantetheine thiol were chemically modified. Pre-treatment of the *holo* protein with DTT followed by complete removal of the excess reducing agent by desalting into a pH5 buffered solution allowed addition of two NPS groups to the protein, but only if the derivatisation was carried out immediately after the desalting process (expected $M_r = 9763$ for 2 additions of NPS; observed $M_r = 9763.2 \pm 1.1$). Thirty minutes after de-

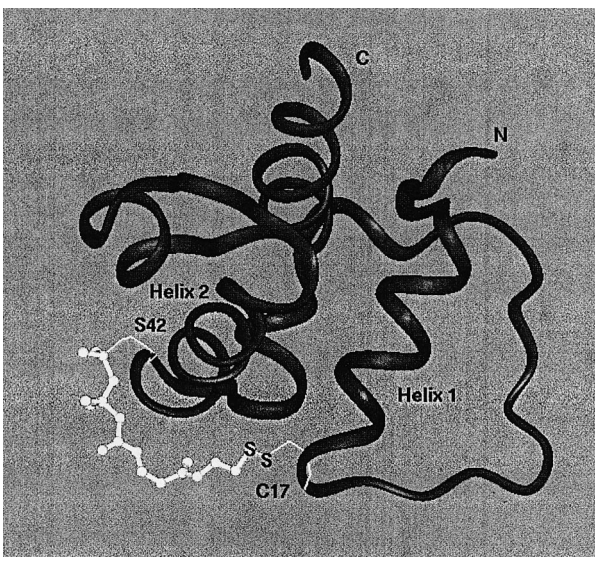

Fig. 2. Ribbon representation of the solution structure of *holo* act ACP showing the disulphide bond between the 4′-phosphopantetheine prosthetic group and cysteine 17. The 4′-phosphopantetheine chain was modelled onto the structure of the ACP using Insight II.

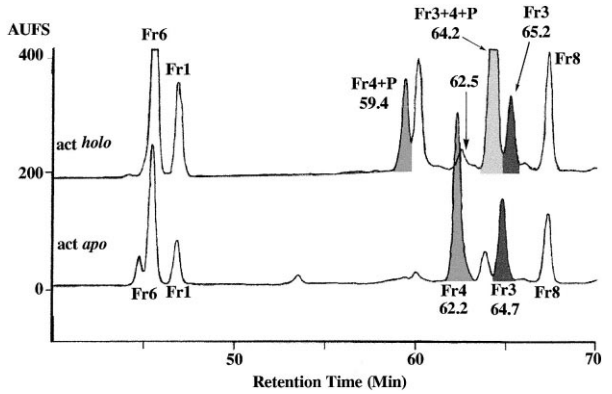

Fig. 3. HPLC analysis of trypsin proteolytic fragments from *holo* act ACP. Fragment 4 elutes earlier for *holo* ACP ($R_t = 59.4$) compared to *apo* ACP ($R_t = 62.2$) due the presence of the 4′-phosphopantetheine group (ESMS analysis F4 + 4′PP expected $M_r = 1122.1$ for (M+2H); observed $M_r = 1122.1 \pm 0.6$). An extra fragment due to fragment 3 coupled to fragment 4 by the 4′-phosphopantetheine group ($R_t = 64.2$) is present only in the *holo* ACP HPLC trace (ESMS analysis expected $M_r = 1514.2$ (M+3H), 1134.9 (M+4H), 908.9 (M+5H); observed $M_r = 1514.1$, 1135.3, 912.6).

salting, the *holo* ACP was again unreactive in the presence of NPDS. When comparing NPS addition to both *holo* and *apo* act ACPs the addition to the 4′-phosphopantetheine thiol occurred slightly faster than to the exposed cysteine thiol. This is possibly due to greater accessibility of the terminal thiol group of the exposed prosthetic group in solution. The relative difference in reactivity of the 4′-phosphopantetheine and cysteine thiols was small, however, suggesting that chemical discrimination between these groups on this basis was not possible.

The results showing no addition of NPS to *holo* act ACP are consistent with the formation of an intramolecular disulphide bond between the 4′-phosphopantetheine and cysteine thiols rendering these groups inaccessible to chemical modification (Fig. 2). To confirm this both *apo* and *holo* act ACP were subjected to tryptic digestion. The resulting fragments were separated by HPLC and analysed by ESMS. Of the predicted eight fragments only the fragment consisting of a single arginine residue (Arg-12) was unidentified.

The elution times and observed masses of fragments 5, 7, 6, 1, 3, and 8 (in order of increasing retention time) are identical for both the *apo* and *holo* proteins, although there is an overall reduction in the area of the peak corresponding to fragment 3 (the fragment containing cysteine 17) in the *holo* digest when compared with that of the *apo* protein (Fig. 3). Significant differences between the HPLC analyses of the *apo* and *holo* ACP digests were only seen with regard to fragment 4 which contains serine 42. Fragment 4 obtained from the *apo* protein incubation and lacking the 4′-phosphopantetheine cofactor, eluted at 62 min. The same fragment isolated from the *holo* ACP incubation and therefore phosphopantetheinylated eluted slightly earlier at 59 min. HPLC analysis of the *holo* digest also included a second peak not present in the *apo* ACP incubation ($R_t = 64.2$ min). ESMS analysis gave *m/z* values of 1514.2, 1134.9 and 908.9 Da (for M+3H, M+4H and M+5H) which correspond exactly to the values predicted for fragments 3 (containing cysteine 17) and 4 linked by the 4′-phosphopantetheine chain.

In contrast to the *holo* act ACP, *holo* otc ACP, which also

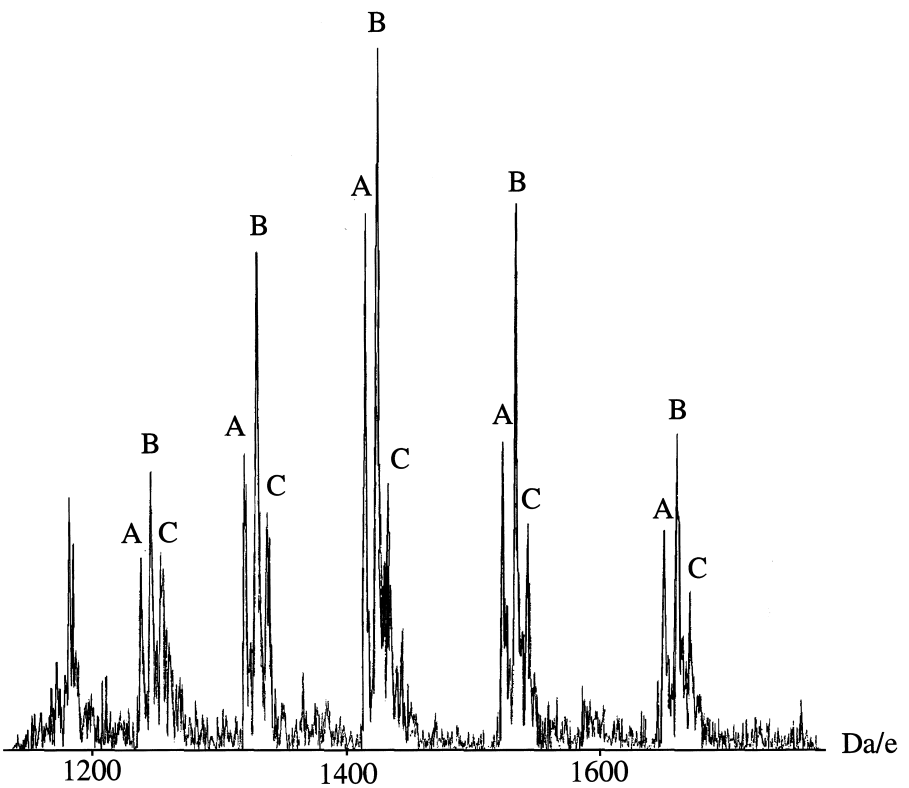

Fig. 4. ESMS spectrum of *holo* gris ACP showing presence of intermolecular dimers. A: Minus methionine dimer $(-\text{Met})_2$, expected $M_r = 19768$, observed = 19770.4 ± 5.7; B: mixed plus-minus methionine dimer $(\pm\text{Met})_2$: expected $M_r = 19899$, observed = 19901.3 ± 2.3; C: plus methionine dimer $(+\text{Met})_2$: expected $M_r = 20030$, observed = 20034.1 ± 2.6.

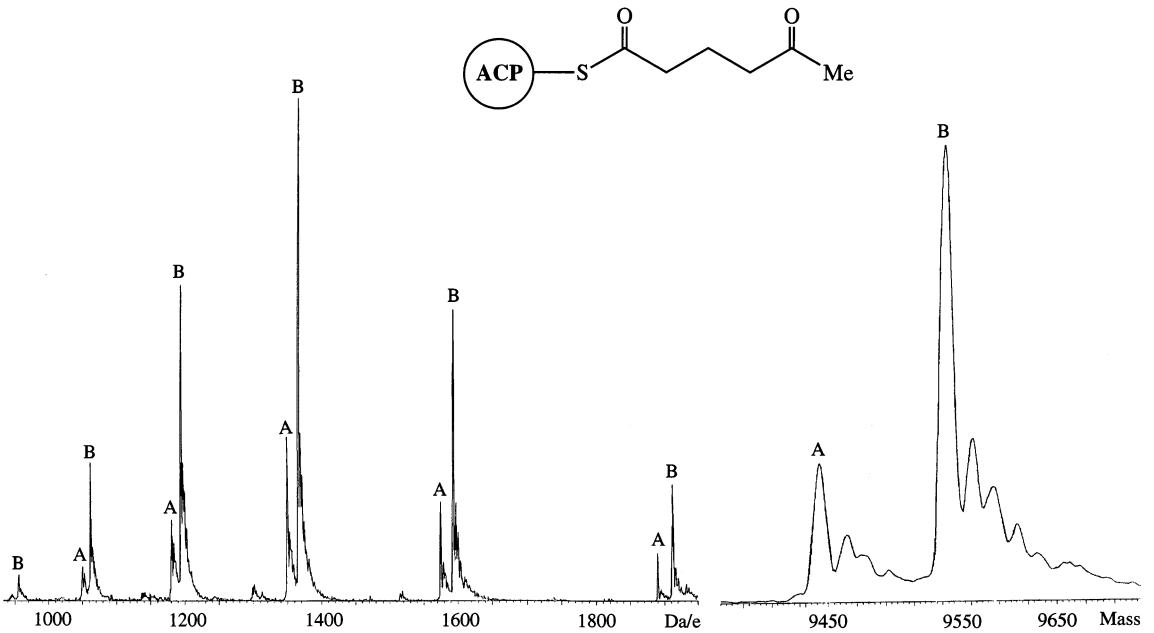

Fig. 5. ESMS spectrum of the reaction of 5′-ketohexanoyl imidazolide with *holo* ACP. A: *holo* act C17S ACP: expected $M_r = 9445$, observed = 9444.6 ± 2.0; B: 5′-ketohexanoyl-*holo* act C17S ACP: expected $M_r = 9557$, observed = 9555.2 ± 0.8.

has an exposed cysteine (Cys-16), did not readily form an intramolecular disulphide. The length of the 4′-phosphopantetheine side chain has been established as 20 Å [2], while the distance between the cysteine (Cys-17) and the serine (Ser-42) bearing the prosthetic group is approximately 12.4 Å for the act protein (unpublished results). By comparison, the distance between cysteine 16 and serine 41 in the otc ACP is 11.2 Å, yet no evidence for formation of an intramolecular disulphide has been observed. The overall global fold for the otc ACP is very similar to that of the act ACP (unpublished results) differing most markedly at the C-terminal end where the otc ACP has an additional 12 amino acid sequence which adopts no definite conformation. Clearly then the development of the intra-molecular disulphide is possible where distance is the only consideration. Its absence in the otc ACP suggest that other factors may prevent its formation. *Holo* gris ACP does not contain any cysteine residues and would not therefore be expected to form an intramolecular disulphide. Three distinct dimers were, however, seen for the *holo* gris protein, corresponding to the *holo* minus methionine dimer, the *holo* plus methionine dimer, and the mixed *holo* plus, *holo* minus methionine dimer (Fig. 4). Such intermolecular dimers form readily and are observable on native gels, but this is the first instance that such dimeric ACP species have been reported to survive the ESMS analysis conditions.

N-Acyl imidazolides have previously been used to chemically acylate the *E. coli holo* FAS ACP [27] and the *S. erythraea* putative *holo* FAS ACP [29]. Such acylations are specific for the 4′-phosphopantetheine (exposed cysteine residues are not present in FAS ACP) and result in acyl ACPs whose physical and biological properties are indistinguishable from enzymically prepared substrates. At equimolar concentrations of hexanoyl imidazolide and ACP, mono acylation of the *apo* act protein was evident within 10 min (expected $M_r = 9216$; observed $M_r = 9220.4 \pm 3.8$). Following DTT treatment, rapid formation of the diacylated derivative of *holo* act ACP was observed (expected $M_r = 9654$; observed $M_r = 9652.2 \pm 0.8$). This suggests that chemical acylation will not distinguish between the exposed cysteine and 4′-phosphopantetheine thiols confirming results obtained using the thiol specific reagent, NPDS. Such chemically formed diacylated species would be of little use either as substrates for other PKS enzymes, or in any studies of the interactions that the acyl chain has with specific amino acids of the protein.

In order to avoid the formation of bis adducts in the chemical acylation studies, or the development of the unreactive cysteine-phosphopantetheine disulphide, cysteine 17 of act ACP was mutated to a serine by site-directed mutagenesis. This mutation did not significantly affect the purification of the act PKS ACP and levels of protein isolated were analogous to those found for the native protein. The C17S mutated ACP could also substitute for the wild type act ACP forming an active minimal PKS system (act ketosynthase, act chain length factor, plus act acyl carrier protein), suggesting that the amino acid mutation has not significantly affected the interactions between the PKS components (unpublished results). ESMS analysis confirmed the single amino acid modification. The *apo* form of the C17S mutated ACP did not react with NPDS (expected $M_r = 9101$; observed $M_r = 9103.1 \pm 2.0$) consistent with the removal of the exposed reactive cysteine thiol, while a single NPS addition to the *holo* form of the C17S ACP was identified (expected $M_r = 9594$; observed $M_r = 9596.8 \pm 0.8$). Similarly, *apo* act C17S ACP did not react with hexanoyl imidazolide at equimolar concentrations, while *holo* act C17S ACP formed a mono-acylated species. This acylated ACP did not react further with the NPDS indicating that acylation had occurred specifically on the thiol of the 4′-phosphopantetheine cofactor.

Chemical derivatisation of the *holo* act C17S ACP with the reduced triketide analogue, 5-ketohexanoyl imidazolide (expected $M_r = 9557$; observed M_r 9555.2 ± 0.8) (Fig. 5), as well as the ethylene ketal protected form of the acetoacetyl diketide, 3-oxo-(1,2-ethanediol acetal)-butanoyl imidazolide (expected $M_r = 9570$; observed $M_r = 9572.5 \pm 2.0$) also proved

successful, with 90% acylation occurring after 15 min. Acetoacetyl ACP is an excellent target for the study of both polyketide and fatty acid biosynthesis as it is the product of the first condensation reaction in both pathways. Although previously synthesised as its imidazolide derivative [30] this compound proved difficult to isolate in useful quantities and consequently the protected form was used in these experiments. Unfortunately incubation of the protected acetoacetyl derivative of the ACP under mild acidic conditions failed to remove the protecting group to yield acetoacetyl ACP. Both *holo* gris and *holo* otc ACPs were also successfully acylated using these acyl imidazolides (Table 1). As expected, in the case of the *holo* otc ACP a double acylation was observed as both cysteine and 4′-phosphopantetheine thiols are present. As was seen for the act ACP the relative difference in reactivity of the otc ACP 4′-phosphopantetheine and cysteine thiols was small. Thus chemical derivatisation methods are capable of specifically acylating polyketide ACPs with functionalised as well as fatty acid intermediates.

The exposed cysteine, which is present in four out of the six type II *Streptomyces* PKS ACPs isolated to date, would not interfere with the specific acylation of the 4′-phosphopantetheine thiol if an acyl transferase were used to catalyze this reaction. This would eliminate the necessity for the modification of an amino acid which could potentially play a role in interprotein interactions within the PKS. No discrete acyltransferase domain has yet been identified in the gene clusters encoding type II PKSs. Malonyl co-enzyme A transacylase genes have, however, been identified in the putative FAS clusters of *S. glaucescens* [31] and *S. coelicolor* [32]. These genes encode enzymes reportedly capable of transferring the malonate of malonyl coenzyme A to both FAS as well as PKS ACPs, but whether these enzymes have a broad enough substrate specificity to prove synthetically useful in PKS ACP acylation is still unknown. Acyl derivatives of FAS ACPs have been synthesised enzymically using acyl ACP synthetase from *E. coli* although yields using short acyl chains ($<C_{12}$) are poor [22]. For this reason chemical derivatisation using short acyl imidazolides has become the method of choice. Acyl ACP synthetase is most efficient at ligating C_{14}-C_{18} fatty acids to FAS ACPs with the retention of stable, native protein structure although the efficacy of this enzyme on PKS ACPs was not known. When commercially available *E. coli* acyl ACP synthetase was used in an attempt to monoacylate wild type *holo* act ACP, *holo* act C17S ACP, as well as *holo* otc ACP with palmitic acid, none of the PKS ACPs examined were enzymically acylated even after prolonged incubation times although in control experiments *E. coli* FAS ACP served as an efficient substrate. This may indicate that although the gross 3 dimensional structures of PKS and FAS ACPs are similar [15], subtle differences exist which may prevent adequate enzyme interactions. Only acylation using palmitic acid has been investigated, however, and therefore the enzyme catalysed transfer of shorter carbon chains lengths cannot be rigorously excluded by these experiments. Recently, however, we have managed to form both malonyl as well as acetoacetyl ACPs directly from both co-enzyme A as well as *N*-acetyl cysteamine derivatives [33]. Initial results suggest that this self catalysed transfer may be a unique property of the polyketide ACPs. This may explain the fact that an active acyl transferase has not been identified in PKS enzyme clusters and may give some insight in to the early events occurring in polyketide biosynthesis.

The ability to specifically acylate PKS ACPs by chemical methods will prove invaluable in studies to determine structural changes induced in the protein by interactions of the acyl chain with specific amino acids within the binding cleft. Using this technique milligram quantities of acyl ACP can be rapidly obtained. Highly functionalised polyketide intermediates may need some form of functional group protection to prevent spontaneous cyclisation or to aid acyl imidazolide formation prior to derivatisation of the protein. Removal of such protecting groups once bound to the protein may prove difficult, however, if the native structure is to remain intact.

Finally, appropriate acyl ACPs will be prepared for use as substrates to probe the specificity of other PKS enzymes. In this way further information on the factors controlling polyketide biosynthesis may be obtained and novel polyketide structures produced in vitro.

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