Post-translational modification of heterologously expressed *Streptomyces* type II polyketide synthase acyl carrier proteins

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Abstract Expression in *Escherichia coli* of *Streptomyces* acyl carrier proteins (ACPs) associated with polyketide biosynthesis using the pT7-7 expression system of Tabor and Richardson led to the production predominantly of inactive *apo*-proteins lacking the 4′-phosphopantetheinyl prosthetic group essential for polyketide synthase activity. Modification of growth conditions led to an increase of production of active *holo*-protein for the actinorhodin (act) ACP, but this technique was ineffective for oxytetracycline (otc) and griseusin (gris) ACPs. Labelling experiments revealed that a low level of otc ACP expressed prior to induction was produced mainly as active *holo*-protein, while post-induction ^{15}N-labelled protein was almost exclusively in the *apo*-ACP form. Limiting endogenous *holo*-acyl carrier protein synthase (ACPS) concentration was implicated as responsible for low *apo*-ACP to *holo*-ACP conversion, rather than limiting substrate (coenzyme A) and cofactor (Mg^{2+}) concentrations. Co-expression of act and gris ACPs with ACPS in *E. coli* led to high levels of production of active *holo*-ACPs and ACPS. We have also made the significant observation that ACPS is able to transfer acylated CoA moieties to act *apo*-ACP.

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Key words: Polyketide; Acyl carrier protein; *Streptomyces*; Heterologous expression; Post-translational modification; 4′-Phosphopantetheine

1. Introduction

Polyketides form a large and diverse group of secondary metabolites produced by plants, fungi, marine organisms and bacteria (Fig. 1) [1]. Amongst bacteria the *Actinomycetes* have been the chief source of pharmacologically active polyketide antibiotics such as oxytetracycline (otc), tetracenomycin (tcm) and erythromycin (ery). The biosynthesis of these compounds and others such as actinorhodin (act) [2] have been studied in depth and shown to closely resemble fatty acid biosynthesis. The carbon backbones of both polyketides and fatty acids are built up by iterative decarboxylative condensations of an acyl chain with acetate derived malonate units [3]. During fatty acid biosynthesis the growing acyl chain is covalently linked to an acyl carrier protein (ACP). The acyl group is bound as a thiol ester to the terminal thiol of a 4′-phosphopantetheine prosthetic group which in turn is covalently bound to the hydroxyl of a conserved serine residue. This is also the case for polyketide biosynthesis where one or more ACPs are required for each polyketide synthase (PKS). In *E. coli* the 4′-phosphopantetheine prosthetic group is attached to the conserved serine of fatty acid synthase (FAS) ACP by *holo*-ACP synthase (ACPS), utilising coenzyme A (CoA) in a Mg^{2+}-dependent reaction [4,5].

As part of ongoing investigations of PKS ACP structure and function, we have been expressing individual PKS genes from the genetic clusters associated with the biosynthesis of act, otc and griseusin (gris). We engineered the first few codons of each *Streptomyces* ACP gene to reflect the codon preferences of *E. coli* (Table 1). Using this strategy each of the genes was expressed in *E. coli*, using the pT7-7 heat-inducible system of Tabor and Richardson [6], to provide sufficient quantities of act, otc and gris ACPs for preliminary characterisation [7]. We have also recently reported preliminary [8] and full [9] tertiary structures of act *apo*-ACP. Although we obtained satisfactory yields of expressed protein (ca 10–30 mg l^{-1}), the ACPs were obtained in predominantly their inactive *apo*-form, lacking the 4′-phosphopantetheine prosthetic group. Active *holo*-ACP formed little (0–10%) of the total purified protein. This low level of *holo*-ACP could be increased in the case of act ACP by increasing the post-induction incubation time from 2 h to 6 h, such that up to 40% of the isolated protein was in the active *holo*-form [7]. However, this increase in the proportion of *holo*-ACP was accompanied by a fall in the total ACP recovered, possibly reflecting proteolytic degradation. In the case of gris and otc ACPs, *holo*-protein is not usually observed under our standard production conditions. Low levels of conversion of *apo* to *holo*-ACP have also previously been observed for expression of tcm ACP in *E. coli* [10].

In order to facilitate our continuing studies of ACP structure and function we set out to investigate the reasons for, and to attempt to remedy, the low conversions of PKS *apo* to *holo*-ACPs when expressed by *E. coli*.

2. Materials and methods

2.1. ^{15}N labelling of otc ACP

E. coli K38 pGP1-2 containing pIJ5231, a derivative of pT7-7 containing *otc-ACP* between initial *Nde*I and terminal *Bam*HI sites, was grown to an A_{595} of 1.5 in 25×100 ml sterile LB medium containing appropriate antibiotics. Cells were spun down (7000 rpm, 15°C, 15 min, Sorval GS3 rotor) and resuspended in 100 ml sterile M9 minimal medium. Cells were again pelleted and resuspended in 100 ml sterile M9 minimal medium. Aliquots of 5 ml were used to inoculate 25×100 ml of sterile M9 minimal medium containing 1.0 g l^{-1} $^{15}NH_4Cl$ and appropriate antibiotics in 500 ml flasks. Induction was performed immediately by heating the flasks to 42°C for 30 min and growth was continued at 30°C for 18 h. Otc ACP was purified as previously described [7].

2.2. Cloning and expression

E. coli strains were DH5α which was used for standard cloning procedures, and K38 containing pGP1-2 (Km^R) used for expression

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of cloned genes. Standard cloning procedures [11] were adopted unless otherwise stated. Expression and purification of ACPs, SDS PAGE and electrospray mass spectrometry were performed as described previously [7]. The synthesis of *acpS* was achieved by polymerase chain reaction (PCR) using *E. coli* DH5α chromosomal DNA as a template, with the following synthetic oligonucleotides: a 37-mer 5′-AGATCTAAGGAGATATACTAATGGCAATATTAGGTTT-3′; and a 26-mer, 5′-AGATCTTTAACTTTCAATAATTACCG-3′. Each oligonucleotide incorporated a *Bgl*II restriction site at its 5′ terminus, and the 37-mer incorporated a ribosome binding site (RBS, underlined) positioned 8 bases upstream from the initial ATG codon. The PCR (100 μl) contained 1 U of Vent DNA polymerase (New England Biolabs), 6 mM $MgSO_4$, 1 pM of each oligonucleotide, a small amount of lysed DH5α and other reagents as specified by the suppliers. The temperature program was 96°C for 60 s, then 55°C for 45 s, then 72°C for 60 s, repeated 25 times, then cooled to 4°C. A PCR product of the expected 408 bp was precipitated from the reaction, then resuspended in water and treated with T4 polynucleotide kinase and ATP at 37°C for 1 h. Phosphorylated DNA was purified by electrophoresis in 2% low melting point agarose, followed by elution according to the method of Boyle and Lew [12] into 25 μl H_2O. The phosphorylated PCR product was ligated into the dephosphorylated *Sma*I site of pBluescript SK(+) using T4 DNA ligase. The sequence of one clone was confirmed by double-strand sequencing using T3 and T7 primers. The cloned gene and RBS were then excised (*Bgl*II) and sub-cloned into the *Bam*HI sites of pIJ2366, a derivative of pT7-7 containing *act*I-ORF3 (C17S), and into pIJ5235, a derivative of pT7-7 containing *gris*-ORF3 [13]. Positive clones were identified by restriction analysis and a positive representative of each was designated pRJC001 (act) and pRJC002 (gris); these were subsequently transformed into the expression host K38 pGP1-2.

2.3. Assay of holo-ACP and ACPS

The viability of the purified act *holo*-ACP was assessed by its ability to act as an acceptor of ^{14}C-labelled malonyl groups from ^{14}C-malonyl CoA catalysed by cloned and expressed (R.J. Cox and T.S. Hitchman, unpublished results) *S. coelicolor* malonyl CoA: ACP transacylase (MCAT) using previously published protocols [7,14].

Holo-ACP synthase activity was measured using the following assay system. *E. coli* strains K38 pGP1-2 and K38 pGP1-2 pRJC001 were grown to A_{595} 1.5 at 30°C in LB medium containing appropriate antibiotics. Induction of *E. coli* K38 pGP1-2 pRJC001 was performed by heating to 42°C for 30 min, followed by incubation at 30°C for 2 h. ACPS containing protein extracts were prepared by breaking the cells (sonication) and removing cell debris by centrifugation. Protein concentration (Bradford) was adjusted to 44 μg μl^{-1} by dilution as necessary. Assays were performed at 37°C for 30 min in a final volume of 20 μl. The assay contained purified act *apo*-ACP (C17S, 50 μM), [^{14}C]acetyl CoA (25 μM, 56 mCi $mmol^{-1}$), Mg^{2+} (10 mM), Tris pH 8.8 (50 mM) and an aliquot (10 μl) of the crude protein fraction containing ACPS. ACP was precipitated by the addition of bovine serum albumin (50 μg) and trichloroacetic acid (50%, 30 μl). Pellets were washed with trichloroacetic acid (50%, 3×50 μl) and resuspended in 2 M Tris (50 μl) then dissolved in scintillant (Packard, 10 ml). The amount of precipitated act [^{14}C]acetyl-*holo*-ACP was determined by scintillation counting for 1 min. Results (Fig. 4) are quoted as an average of three parallel assays.

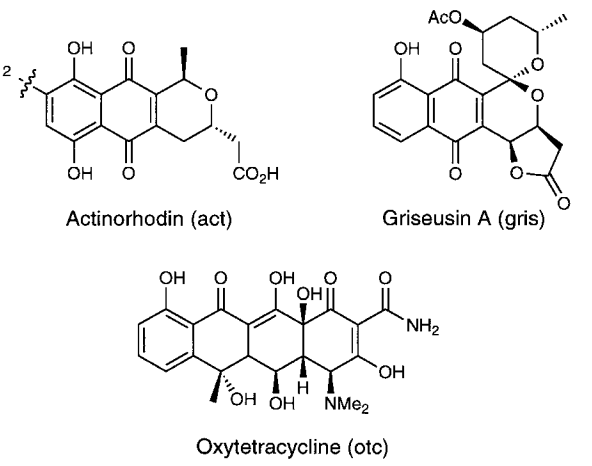

Fig. 1. Structures of aromatic polyketides produced by type II polyketide synthases.

3. Results and discussion

Our previous work has shown that wild-type act *holo*-ACP is prone to dimerisation through disulphide formation of cysteine-17 [7]. This residue also forms an internal disulphide with the terminal thiol of the 4′-phosphopantetheine prosthetic group (J. Crosby, K. Byrom and H.C.O Brown, unpublished results). These problems were overcome by replacing cysteine-17 with serine. The C17S mutant was expressed from pIJ2366 (R. Cox, J. Crosby and K. Byrom, unpublished results). This protein is expressed at similar levels (10–15 mg l^{-1}) to the wild-type act ACP in *E. coli*. The protein was purified from *E. coli* K38 pGP1-2 pIJ2366 by initial ammonium sulphate precipitation. A sodium chloride gradient was then used to elute bound proteins from a Q-Sepharose anion exchange FPLC column (Pharmacia). ACP was eluted as two peaks at approximately 500 mM NaCl (Fig. 2A). Analysis of the major peak by electrospray mass spectrometry (ESMS) gave a mass of 9101 Da indicative of act *apo*-ACP minus the N-terminal methionine residue (Fig. 2C, Table 2). Similar analysis of the minor peak (10–15% of the major peak) gave a mass of 9444 Da corresponding to act *holo*-ACP, again minus the N-terminal methionine (Fig. 2D). Otc and gris ACPs were

Table 1
Plasmids and *E. coli* strains used in this study

Plasmid or strain	Description	Reference or source
E. coli		
K38	HfrC(λ)	[6]
DH5α	*supE44 ΔlacU169* (ϕ80 *lacZΔM15*) *hsdR17 recA1 endA1 gyrA96 thi-1 relA1*	[11]
Plasmids		
pGP1-2	Helper plasmid to each of the below	[6]
pIJ5232	*actI* ORF3 under control of T7 gene 10 promoter	[7]
pIJ2366	derivative of pIJ5232 containing C17S mutation	[a]
pIJ5235	*gris* ORF3 under control of T7 gene 10 promoter	[b]
pIJ5231	*otc*-ACP gene under control of T7 gene 10 promoter	[7]
pRJC001	pIJ2366 containing *acpS* inserted inserted at *Bam*HI	This study
pRJC002	pIJ5235 containing *acpS* inserted inserted at *Bam*HI	This study

[a]R. Cox, J. Crosby and K. Byrom, unpublished results.
[b]Kind gift of Mrs M.J. Bibb, John Innes Centre, Colney Lane, Norwich, UK.

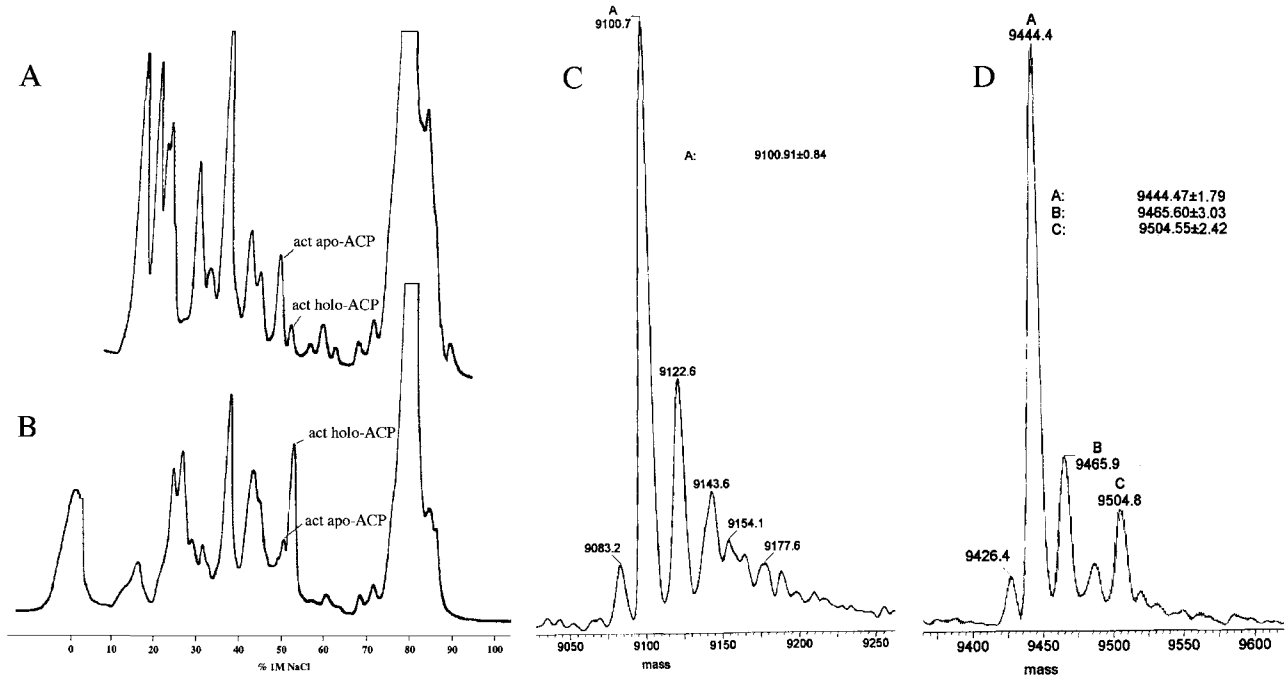

Fig. 2. Purification and ESMS analysis of act ACP. A: Q-Sepharose FPLC trace of *E. coli* protein fraction containing act ACP expressed from pIJ2366. B: Q-Sepharose FPLC trace of *E. coli* protein fraction containing act ACP expressed from pRJC001. C: ESMS of act *apo*-ACP, note distinctive envelope of Na^+ adducts. D: ESMS of act *holo*-ACP, again showing Na^+ adducts.

similarly expressed from pIJ5231 and pIJ5235. Under conditions comparable to the act ACP expression, *apo*-gris and *apo*-otc ACPs were produced with no observable amounts of the *holo*-form of either. Thus the ability of the expressed ACPs to act as substrates for the endogenous *E. coli* ACPS appeared to be limited.

One possible cause of low ACP conversion was low intracellular concentrations of the substrate (CoA) and cofactor (Mg^{2+}) required by ACPS. A similar effect has been observed by Chapman-Smith et al. who found that ATP concentrations were limiting for the conversion of highly expressed *apo*-biotin carboxy carrier protein to its biotinylated *holo*-form by biotin ligase in *E. coli* [15]. We thus examined whether the supplementation of additional substrate and cofactor, CoA and Mg^{2+} respectively, to a cell-free extract of heat-shocked *E. coli* K38 pGP1-2 pIJ2366 could facilitate conversion of the act *apo*-ACP to the *holo*-form. Thus lysed suspensions of the induced strain in the presence of protease inhibitors were incubated at 37°C with added CoA (50–250 μM) and Mg^{2+} (5–25 μM) for 30 min. Subsequent purification of act ACP in the usual way revealed no increase in the concentration of *holo*-ACP with respect to controls. Thus it appeared that intra-cellular substrate CoA and Mg^{2+} concentrations were not limiting for the conversion of heterologous PKS *apo* to *holo*-ACPs in *E. coli*. The results of Leadlay et al. would appear to support this hypothesis. They showed that the heterologous expression of a presumed FAS ACP from the Actinomycete *Saccharopolyspora erythrae* in *E. coli* yielded high levels (20 mg l^{-1}) of predominantly (65%) *holo*-ACP [16,17]. Clearly in this case CoA and Mg^{2+} cannot be limiting. This is in agreement with the recent observations of Cronan et al. who showed that under usual conditions in vivo CoA concentrations are high enough to ensure full conversion of endogenous *E. coli* ACP to *holo*-protein [18].

Table 2
Calculated and observed molecular weights of ACPs

ACP[a]		Molecular weight (Da)	
		Calculated	Observed
act C17S (−met)	*apo*	9100.9	9101±0.8
	holo	9440.9	9444±1.8
gris	*apo*	9675.6	9678±3.8
	holo	10015.6	10020±4.3
gris (−met)	*apo*	9544.4	9549±4.0
	holo	9884.4	9879±4.0
otc	*apo*	9915.9	9914±3.1
	holo	10255.9	10255±1.3
^{15}N-otc	*apo*	10030.9	10026±3.8
	holo	10370.9	10367±3.8

[a]ACPs are observed to have retained their initial methionine residues unless otherwise indicated.

We obtained further information about the activity of ACPS as a result of an experiment to produce quantities of otc ACP highly enriched in ^{15}N for NMR studies. Thus we grew *E. coli* K38 pGP1-2 pIJ5231 in LB medium at 30°C to mid-log phase. The cells were washed, then transferred to M9 minimal medium containing $^{15}NH_4Cl$, and immediately induced by heat shock to 42°C for 30 min. Subsequent growth at 30°C for 18 h, followed by purification of otc ACP, revealed that two otc ACP peaks eluted from the Q-Sepharose FPLC column (Fig. 3A). These were analysed by ESMS. As expected the first peak contained otc *apo*-ACP (18 mg l^{-1}) in ^{15}N-labelled form with mass of 10026 Da (10031 Da predicted for all 115 N-positions ^{15}N-labelled, Fig. 3B). This fraction contained a trace amount (<1% of the major peak) of unlabelled *apo*-ACP (measured mass 9914 Da, calcu-

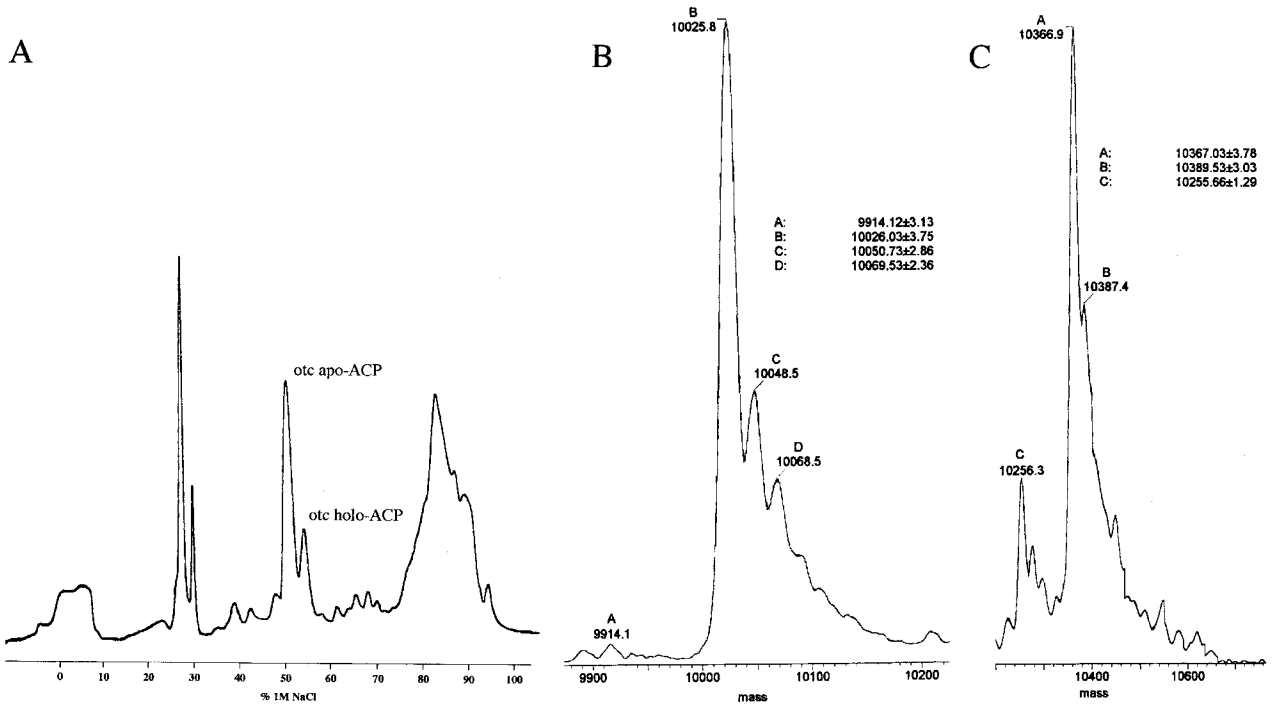

Fig. 3. Purification and ESMS analysis of otc ACP after growth and induction in minimal medium containing $^{15}NH_4Cl$. A: Q-Sepharose FPLC trace of *E. coli* protein fraction containing otc ACP expressed from pIJ5231. B: ESMS of [^{15}N]otc *apo*-ACP, note distinctive envelope of Na^+ adducts. C: ESMS of [^{15}N]otc *holo*-ACP, again showing Na^+ adducts as well as a significant proportion of unlabelled otc *holo*-ACP.

lated 9916 Da). The second fraction eluted from the FPLC column contained labelled otc *holo*-ACP (4 mg l^{-1}) with a mass of 10 367 Da (10 376 Da predicted for all 115 N positions labelled, Fig. 3C). Significantly this species contained approximately 10% (ca 0.4 mg l^{-1}) of a species of otc *holo*-ACP containing no ^{15}N label with a mass of 10 256 Da (10 256 Da predicted for otc *holo*-ACP).

The long post-induction period, possibly coupled with a difference in media composition, led in this case to the production of otc *holo*-ACP at a similar level (10%) to that observed for the production of act *holo*-ACP under short (2 h) post-induction conditions. Significantly however, since labelling and induction were performed simultaneously, unlabelled protein must have been produced prior to addition of label and concomitant induction. Of the unlabelled protein, ca 0.18 mg l^{-1} was *apo*-ACP, while ca 0.4 mg l^{-1} was *holo*-ACP. At least 60% of the protein produced prior to induction was therefore *holo*-ACP, contrasting with only 10% *holo*-ACP formed after induction. This observation may possibly be interpreted as evidence for substrate inhibition of ACPS, since

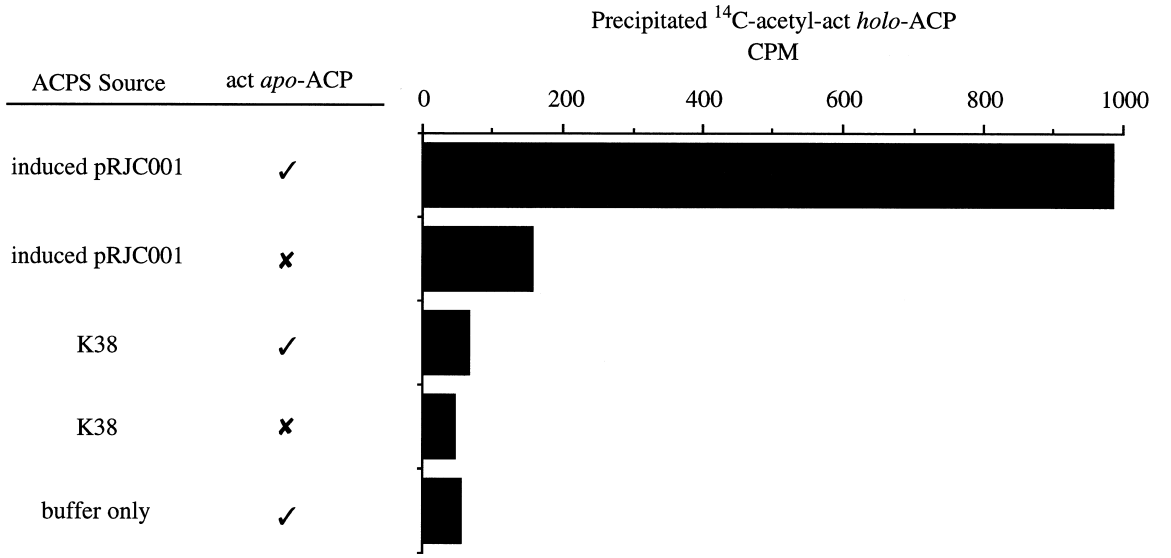

Fig. 4. Results of ACPS activity assay using purified act *apo*-ACP, [^{14}C]acetyl CoA and cell free extracts of the indicated *E. coli* strains. Conditions are described in Section 2.

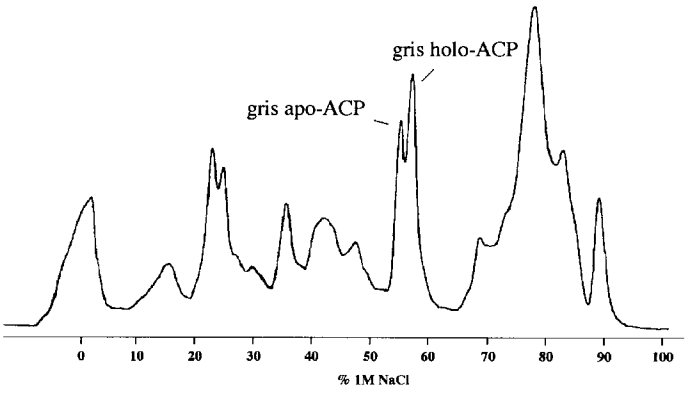

Fig. 5. Q-Sepharose FPLC trace of *E. coli* protein fraction containing gris ACP expressed from pRJC002.

ACPS appears to be able to convert the low concentration of otc ACP (ca 0.4 mg l^{-1}) produced prior to induction, but was unable to cope with high levels (ca 18 mg l^{-1}) of otc *apo*-ACP expressed after induction. This observation is consistent with that of Walsh et al. who have isolated the *E. coli* ACPS and shown that *E. coli apo*-ACP binds tightly to ACPS with a submicromolar K_m [19,20]. Indeed, substrate inhibition was observed at concentrations of *apo*-ACP above 2 μM.

Our results, and those of Hutchinson [10], show that the type II PKS ACPs are clearly poor in vivo substrates for the endogenous *E. coli* ACPS. This contrasts with the *S. erythrae* FAS ACP which appears to be a relatively good in vivo substrate of *E. coli* ACPS [16,17]. In addition otc ACP could also cause substrate inhibition. We reasoned that an increase of in vivo ACPS concentration should allow partial or full conversion of *apo*- to *holo*-type II PKS ACPs. The expression vectors pIJ2366 and pIJ5235 contain *act*I-ORF3 from *Streptomyces coelicolor* and *gris*-ORF3 from *S. griseus* respectively between initial *Nde*I and terminal *Bam*HI sites, downstream from T7 promoters. Construction of operons containing ACP ORFs and *acpS* should ensure synthesis of both proteins at similar levels, thus increasing the rate of *apo*- to *holo*-ACP conversion and overcoming any potential substrate inhibition of endogenous ACPS. Walsh and co-workers have recently reported the cloning of the *E. coli* ACPS gene, *acpS* (previously *dpj*) [19,20]. A synthetic *acpS* was therefore constructed in which the leading and trailing sequences contained convenient *Bgl*II restriction sites. The leading sequence also contained a ribosome binding site 8 base-pairs upstream of the initial ATG codon. This construct was inserted at the *Bam*HI sites of pIJ2366 and pIJ5235 to create pRJC001 and pRJC002 respectively. Restriction analysis confirmed that the cloning had occurred in the correct direction.

The expression of pRJC001 and pRJC002 in *E. coli* K38 pGP1-2 was performed as usual. For pRJC001 SDS PAGE analysis (data not shown) of induced and uninduced cultures showed the presence of two proteins of apparent M_w 14 kDa and 4 kDa corresponding to ACPS and act ACP respectively in the induced lysates. Purification of act ACP from an induced culture in the usual way again gave two peaks from the Q-sepharose column corresponding to act ACP (Fig. 2B). ESMS analysis of these fractions indicated that the ACP was now present in >90% *holo*-form. Overall total protein synthesis mirrored the previously observed concentration of ca 10–15 mg l^{-1}. The two proteins expressed from pRJC001 were assayed to ensure that activity was retained using this system. Firstly the ability of purified act *holo*-ACP to accept ^{14}C-labelled malonyl groups from [^{14}C]malonyl CoA, catalysed by purified *Streptomyces coelicolor* MCAT [7,14], was confirmed. Secondly we examined the activity of ACPS, using a modified version of the assay of Vagelos et al. [4,5]. This assay consists of monitoring the transfer of ^{3}H-labelled phosphopantetheine from CoA to *apo*-ACP. Crude protein fractions containing ACPS were obtained from *E. coli* K38 pGP1-2 and from induced *E. coli* K38 pGP1-2 pRJC001. ^{3}H-Labelled CoA was unavailable to us, and we reasoned that ACPS was unlikely to discriminate the distal thiol group of CoA and may utilise [^{14}C]acetyl CoA with similar efficiency. We were pleased to observe that acetyl CoA is indeed a substrate for ACPS in an assay containing ACPS, purified act *apo*-ACP and [^{14}C]acetyl CoA. Considerably higher activities of ACPS were observed in lysates from induced *E. coli* K38 pGP1-2 pRJC001 than from the control strain (Fig. 4). Although the protein extract isolated from the induced culture of *E. coli* K38 pGP1-2 pRJC001 was some 8-fold more active than the control strain in the ACPS assay, only 0.8% of the available act *apo*-ACP was converted to act [^{14}C]acetyl-*holo*-ACP under the assay conditions.

Similar expression of pRJC002 followed by standard purification revealed two protein peaks eluted from the Q-Sepharose FPLC column in approximately 2:3 ratio (Fig. 5). Analysis of the peaks by SDS PAGE and ESMS (data not shown) confirmed that they were *holo*- and *apo*-forms of gris ACP. Interestingly ESMS analysis also revealed that each species was a 1:1 mixture of forms with and without the N-terminal methionine. In accord with our previous observations total gris ACP concentration was ca 6–8 mg l^{-1}. Attempts to increase the proportion of gris *holo*-ACP through increased post-induction time were successful. However, as we have previously observed, longer post-induction times lead to significant loss of total ACP protein.

These results indicate that increased levels of ACPS in vivo can overcome the slow transformation of heterologous PKS *apo*-ACPs to the phosphopantetheinylated *holo*-form, supporting our hypothesis that PKS ACPs are poor substrates of the endogenous *E. coli* ACPS. In particular the ^{15}N labelling experiment suggests that high levels of expressed heterologous *apo*-ACPs may also inhibit endogenous ACPS in agreement with the previous observations of Walsh et al. with *E. coli* FAS-ACP [19,20]. Although over 90% of act ACP was converted to *holo*-ACP when co-expressed with ACPS, it appeared that under similar conditions gris ACP was more

poorly converted. However, this is the first time that we have observed gris *holo*-ACP. This technique represents a significant advance in our ability to produce active *holo*-proteins for our continuing studies of PKS ACP structure and function. We have also made the significant observation that ACPS is able to transfer acylated CoA moieties to act *apo*-ACP. This methodology is currently being applied to the production of other PKS *holo*-ACPs, and exploitation of the ability of ACPS to transfer acylated phosphopantetheine moieties to *apo*-ACPs, as a route to the study of the 3D structure of acylated *holo*-ACPs, is being pursued.

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