Ability of *Streptomyces* spp. acyl carrier proteins and coenzyme A analogs to serve as substrates *in vitro* for *E. coli* holo-ACP synthase

Amy M Gehring[1], Ralph H Lambalot[1], Kurt W Vogel[2], Dale G Drueckhammer[2] and Christopher T Walsh[1]

Introduction: The polyketide natural products are assembled by a series of decarboxylation/condensation reactions of simple carboxylic acids catalyzed by polyketide synthase (PKS) complexes. The growing chain is assembled on acyl carrier protein (ACP), an essential component of the PKS. ACP requires posttranslational modification on a conserved serine residue by covalent attachment of a 4′-phosphopantetheine (P-pant) cofactor to yield active holo-ACP. When ACPs of *Streptomyces* type II aromatic PKS are overproduced in *E. coli*, however, typically little or no active holo-ACP is produced, and the ACP remains in the inactive apo-form.

Results: We demonstrate that *E. coli* holo-ACP synthase (ACPS), a fatty acid biosynthesis enzyme, can catalyze P-pant transfer *in vitro* to the *Streptomyces* PKS ACPs required for the biosynthesis of the polyketide antibiotics granaticin, frenolicin, oxytetracycline and tetracenomycin. The catalytic efficiency of this P-pant transfer reaction correlates with the overall negative charge of the ACP substrate. Several coenzyme A analogs, modified in the P-pant portion of the molecule, are likewise able to serve as substrates *in vitro* for ACPS.

Conclusions: *E. coli* ACPS can serve as a useful reagent for the preparation of holo-forms of *Streptomyces* ACPs as well as holo-ACPs with altered phosphopantetheine moieties. Such modified ACPs should prove useful for studying the role of particular ACPs and the phosphopantetheine cofactor in the subsequent reactions of polyketide and fatty acid biosynthesis.

Addresses: [1]Department of Biological Chemistry and Molecular Pharmacology, Harvard Medical School, 240 Longwood Avenue, Boston, MA 02115, USA and [2]Department of Chemistry, Stanford University, Stanford, CA 94305, USA.

Correspondence: Christopher T Walsh
E-mail: walsh@walsh.med.harvard.edu

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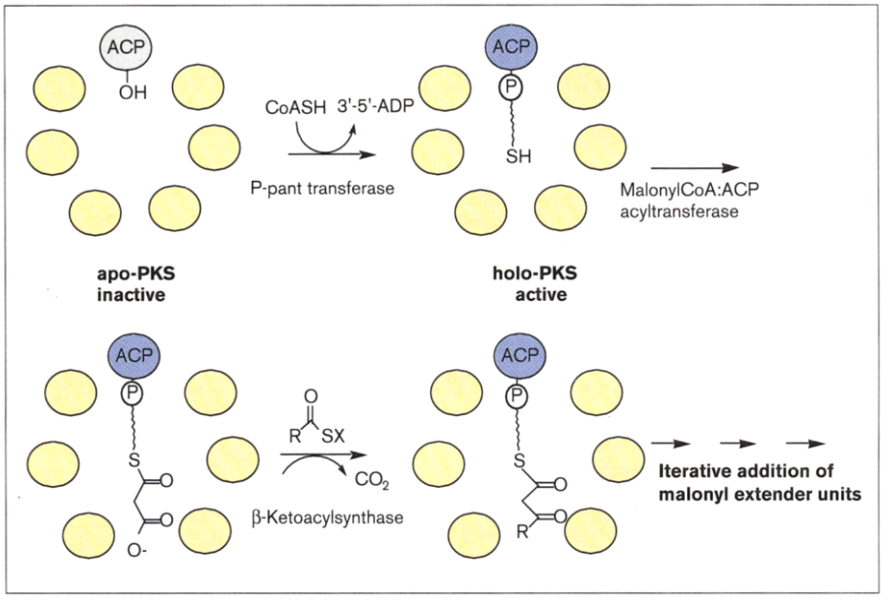
Introduction

Polyketide antibiotics are synthesized either by enzymes of very high molecular weight with multiple catalytic domains in a single polypeptide chain, type I synthases (exemplified by 6-deoxyerythronolide B synthase), or by a multienzyme complex in which the required catalytic activities reside on discrete subunits, the type II synthases (exemplified in *Streptomyces* species aromatic polyketide biogenesis) [1,2]. In both cases, the growing carbon skeleton is elongated as an acyl-S-enzyme species where the sulfur is part of the 4′-phosphopantetheine prosthetic group attached to an acyl carrier protein (ACP) module. In type I polyketide synthases (PKSs), the ACP module is an integral, constituent domain of a larger multidomain polypeptide. In type II synthases, there is a separate ACP of 8–10 kDa that associates with the other enzymatic subunits of the synthase complex (Fig. 1). Thus, in *Streptomyces* spp. producing multiple aromatic polyketides there will be a separate, dedicated ACP for each gene cluster expressed during antibiotic production [2]. These polyketide-specific ACPs are additional to the ACP involved in fatty acid biosynthesis in these bacteria [3,4]. It has been unknown to what extent utilization of a specific ACP is determined by protein–protein recognition in type II PKS complex assembly or by selective posttranslational conversion of an inactive apo-ACP to the phosphopantetheinyl-containing holo-ACP, which is competent for acylation and chain elongation.

There have been mixed reports on whether *Streptomyces* type II PKS apo-ACPs can be posttranslationally modified to produce holo-ACP during heterologous expression in *Escherichia coli* [5,6]. If such holo-ACP formation by the *E. coli* phosphopantetheinyl transferases is of very low stoichiometry, it will be a limiting factor in polyketide biosynthesis in *E. coli*. We have recently reported the cloning, overproduction, purification and characterization of the *E. coli* enzyme holo-acyl carrier protein synthase (ACPS) that catalyzes apo to holo posttranslational modification from cosubstrate coenzyme A (CoASH), using the fatty acid apo-ACP as substrate (Fig. 2) [7]. *E. coli* ACPS is thus a useful reagent with which to assess the ability to recognize and modify *Streptomyces* type II PKS ACPs. Here, we report the ability and catalytic efficiency of

Figure 1



Schematic of the requirement of ACP phosphopantetheinylation for polyketide synthase activity. Conversion of apo-ACP (gray) to holo-ACP (blue) provides the sulfhydryl group through which the intermediates of polyketide biosynthesis are covalently attached. Yellow circles represent the other protein subunits of the type II polyketide synthase which include at least a β-ketoacyl synthase (condensing enzyme) and a chain-length determining factor, and typically also include β-ketoreductases, acyltransferases, dehydrases and cyclases. These enzymes catalyze the iterative addition of malonyl units followed by decarboxylation and condensation reactions, reductions, and cyclizations by which the growing polyketide chain is assembled and modified.

E. coli ACPS to posttranslationally modify the purified apo-ACPs involved in the biogenesis of granaticin by *S. violaceoruber*, of frenolicin by *S. roseofulvus*, of oxytetracycline by *S. rimosus*, and of tetracenomycin by *S. glaucescens* (Fig. 3). We also observe that CoASH analogs including desulfo-CoA and the dethio (carba) analog of acetyl-CoA can be loaded onto apo-ACP by the *E. coli* ACPS.

Results

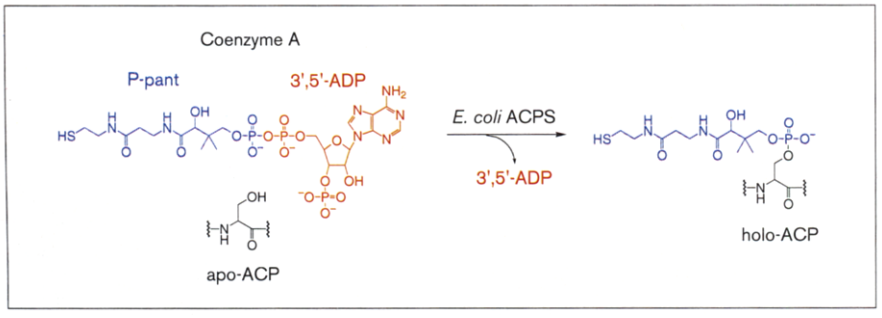
Overproduction of apo-forms of *Streptomyces* spp. ACPs

The PKS ACPs involved in the synthesis of the aromatic polyketide antibiotics granaticin (gra), frenolicin (fren), oxytetracycline (otc), and tetracenomycin (tcm; see Fig. 3) were overproduced in *E. coli*. The tcm ACP was overproduced with a carboxy-terminal hexahistidine (His_6) tag; all the other ACPs were expressed in their native form. Overexpression of these *Streptomyces* ACPs in *E. coli* yielded predominantly apo-ACP that had not been modified by the covalent attachment of 4′-phosphopantetheine as previously reported [5,6]. Mass spectral analysis of these ACPs following the purification step on Q-Sepharose showed no significant levels of holo-protein (data not shown). The *Streptomyces* apo-ACPs were purified in several milligram quantities to near homogeneity in two steps using anion-exchange chromatography on Q-Sepharose followed by perfusion chromatography on POROS 20HQ; the His_6-tagged tcm ACP was also further purified on a nickel-chelate resin (Fig. 4). Protein sequencing of the 10 amino-terminal residues of each ACP revealed that the amino-terminal methionine had been cleaved from gra, fren, and tcm ACP but not otc ACP.

Streptomyces apo-ACPs are modified by *E. coli* holo-ACPS

Although it has previously been shown that the gra, fren, otc and tcm ACPs are not phosphopantetheinylated well *in vivo* when overexpressed in *E. coli* [5,6], the ability of *E. coli* holo-ACPS to catalyze phosphopantetheinyl transfer to these ACPs *in vitro* has not been investigated. Since ACP phosphopantetheinylation is required for the formation of an active polyketide synthase, *E. coli* ACPS may

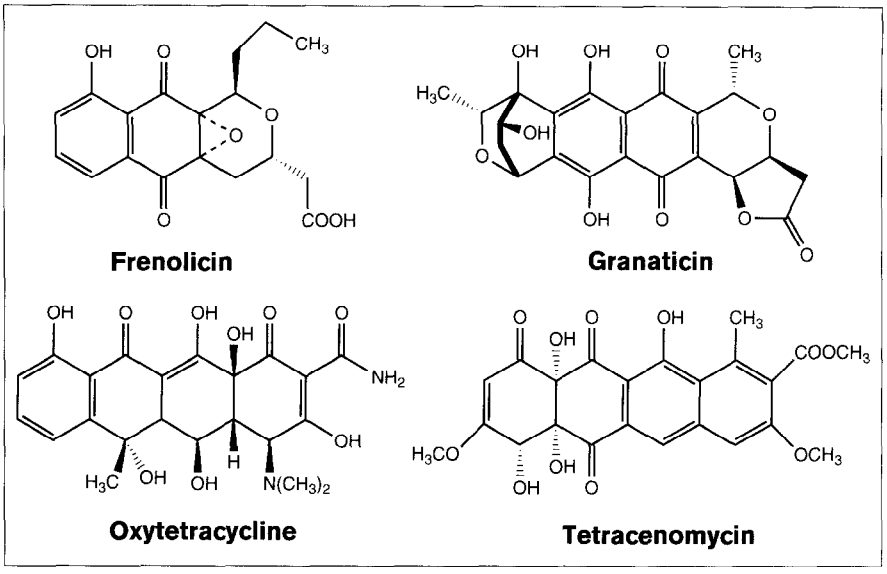
Figure 2



General reaction catalyzed by *E. coli* holo-ACPS. ACPS catalyzes transfer of phosphopantetheine (blue) from CoASH to a conserved serine residue in the apo-ACP substrate to yield 3′,5′ ADP (red) and active holo-ACP in a magnesium-dependent reaction.

Figure 3

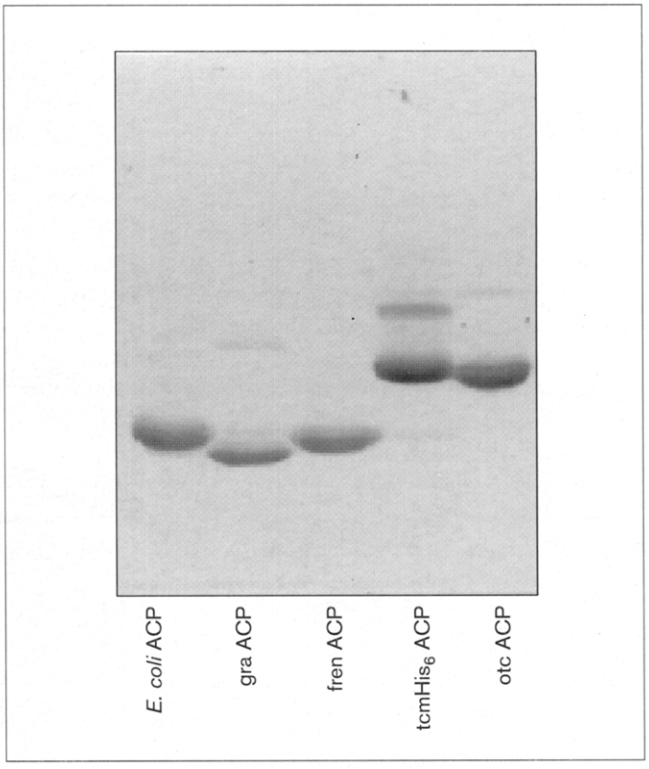
Chemical structures of selected aromatic polyketide antibiotics produced by *Streptomyces* spp. Frenolicin (fren) from *S. roseofulvus*, granaticin (gra) from *S. violaceoruber*, oxytetracycline (otc) from *S. rimosus*, and tetracenomycin (tcm) from *S. glaucescens*. Apo-ACPs from each of their respective polyketide synthases were purified (Fig. 4).



serve as a useful reagent for the preparation of holo-PKS ACPs that can be used to study downstream reactions in polyketide biosynthesis. Mass spectral data were collected for each PKS ACP following incubation for several hours with CoASH and pure ACPS. Table 1 shows that gra, fren, otc, and tcmHis$_6$ ACP were all modified from the apo- to the holo-form as indicated by an increase in mass of ~339 Da due to the addition of phosphopantetheine; modification appeared essentially complete, as indicated by the absence of a mass spectral peak corresponding to the apo-form. It has previously been shown that apo- and holo-forms of *E. coli* ACP [8,9] and *Streptomyces* type II ACPs [6] may be separated by high performance liquid chromatography (HPLC). Using otc ACP as a representative *Streptomyces* PKS ACP, the apo- and holo-forms were similarly resolved (Fig. 5); coinjection of otc apo-ACP and otc ACP following incubation with CoASH and ACPS shows two peaks at 32.6 min and 33.9 min, indicative of the transformation of otc ACP from the apo- to the holo-form and corroborating the mass spectral data.

To investigate the substrate specificity of *E. coli* ACPS, K_m and k_{cat} parameters were determined for each of the *Streptomyces* ACP substrates under initial velocity conditions (Table 2; Fig. 6). When gra ACP was the substrate, the reaction showed severe substrate inhibition of similar magnitude to the substrate inhibition observed with the cognate substrate of ACPS, the ACP of *E. coli* fatty acid synthase (FAS) ([7]; R.S. Flugel, V.L. Healy, R.H.L. and C.T.W., unpublished data). The severity of the observed inhibition of ACPS by gra apo-ACP made an accurate estimation of the K_m and k_{cat} values difficult; gra ACP, however, qualitatively gives the highest V_{max} and as a substrate for ACPS seems to be no more than 10-fold worse

Figure 4



Native PAGE gel (20%) demonstrating the purity of the *Streptomyces* apo-ACPs used. Approximately 10–12 μg of each ACP was loaded. The upper band in the gra, otc and tcmHis$_6$ lanes most probably represents an apo-ACP dimer which has been previously reported [6], given that holo-ACP was not detected by mass spectrometry, that each of these proteins contains a single cysteine residue which is lacking in *E. coli* and fren ACP and that the intensity of this band may be diminished by incubation with DTT.

Table 1

Mass spectral data demonstrating the modification of *Streptomyces* ACPs by *E. coli* ACPS.

Substrate	Molecular mass (Da)			
	Before incubation with ACPS (apo)		After incubation with ACPS (holo)	
	Calculated	Observed	Calculated	Observed
gra ACP	8860	8863	9199	9199
fren ACP	8664	8682	9003	9009
otc ACP	9917	9927	10256	10260
tcm ACP (His_6)	10018	10020	10357	10376

than the *E. coli* ACP. When fren ACP is used as the substrate, the substrate inhibition observed is considerably less than that for gra ACP; there is no substrate inhibition when tcmHis_6 and otc ACP are used. This attenuation of substrate inhibition is concomitant with a decrease in the catalytic efficiency of ACPS modification, with both K_m and k_{cat} kinetic parameters affected. Fren ACP is an approximately four-fold poorer substrate for ACPS than gra ACP, while otc and tcmHis_6 ACP have catalytic efficiencies decreased almost 25-fold when compared to gra ACP and about six-fold when compared to fren ACP. The poorer *Streptomyces* apo-ACP substrates are processed at a ~200-fold lower catalytic efficiency than *E. coli* FAS apo-ACP. The decrease in catalytic efficiency of a *Streptomyces* PKS ACP as a substrate for *E. coli* holo-ACP synthase appears to correlate with a decrease in the overall negative charge in the highly anionic ACP family (Table 2). It should be noted that the carboxy-terminal hexahistidine tag on the tcmHis_6 substrate may well contribute to an increase in its K_m value and/or a decrease in k_{cat} relative to the native tcm ACP substrate, which was not examined here.

Processing of CoASH analogs by holo-ACP synthase

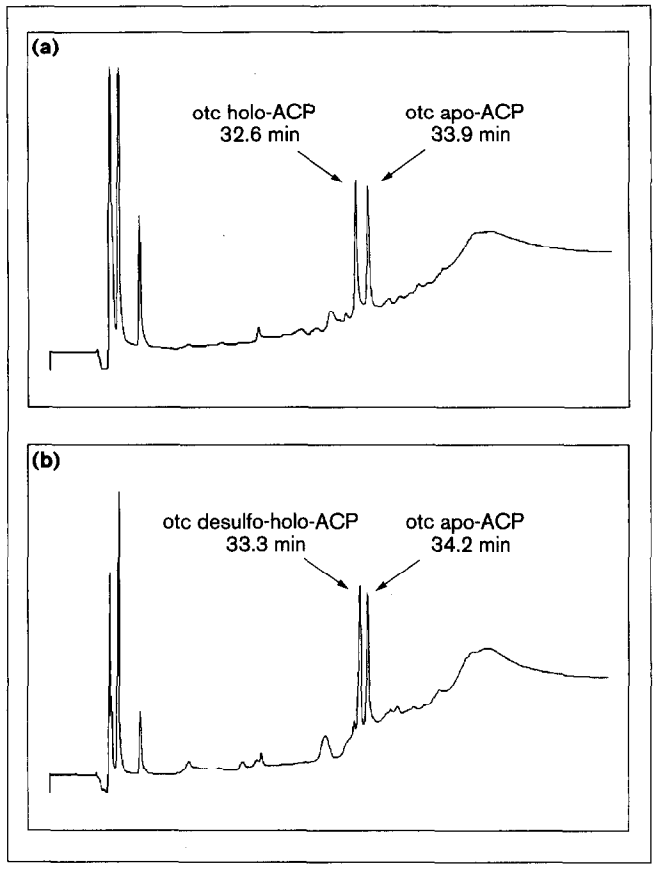
The specificity of *E. coli* holo-ACPS for its coenzyme A substrate was also examined in endpoint assays. CoASH analogs with modifications in the phosphopantetheine portion of the molecule were tested including homocysteamine-CoA, acetonyldethio-CoA, and desulfo-CoA (Fig. 7). ACPS could catalyze modification of *E. coli* ACP with each of these phosphopantetheine analogs as determined by mass spectral analysis (Table 3) and analytical HPLC analysis (Fig. 8). Both the acetonyldethio-holo-ACP and the homocysteamine-holo-ACP could be well resolved from apo-ACP when coinjected onto the HPLC column (Fig. 8). The ability to use desulfo-CoA also extended to the modification of otc ACP as can be seen by its conversion to the desulfo form of the holo-protein (Fig. 5). The conversion of both the carba (dethia) analog of acetyl-CoASH and the homocysteamine form of CoASH to the holo-ACP form (Fig. 8) indicates that ACPS has relaxed specificity for the distal end of the phosphopantetheinyl moiety of CoASH, and suggests that it may be possible to load acylpantetheines directly onto apo-ACP substrates.

Table 2

Kinetic parameters for *Streptomyces* ACPs as substrates for *E. coli* ACPS.

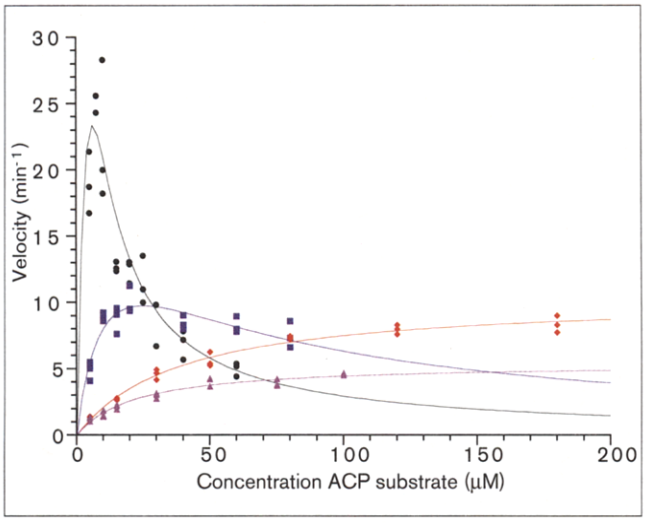
Substrate	Charge (pH 7)	k_{cat} (min^{-1})	K_m (μM)	k_{cat}/K_m ($\mu M^{-1}min^{-1}$)
E. coli ACP	−14.89	80–100	2 (K_i=2)	50
gra ACP	−14.09	≥30	≥5 (K_i=0.6)	6
fren ACP	−12.07	19	12 (K_i=54)	1.6
tcm ACP (His_6)	−11.76	5.4	22	0.25
otc ACP	−11.10	10	39	0.26

Figure 5



Conversion of otc apo-ACP to holo-ACP (220 nm, absorbance units full scale (AUFS) =0.25). **(a)** Otc (50 μM) was incubated as described in the Materials and methods section with CoA in the presence of ACPS and also in the absence of ACPS. Equal volumes (150 μl) of the two assays were then mixed and 200 μl of this mixture was injected onto the HPLC C18 reversed-phase column; Rt (apo-ACP) 33.9 min, (holo-ACP) 32.6 min. Single injections (data not shown) confirmed that the otc apo-ACP was quantitatively converted to the holo-form. **(b)** Sample was prepared as described above except otc apo-ACP was incubated with desulfo-CoA; Rt (apo-ACP) 34.2 min, (desulfo-holo-ACP) 33.3 min. Single injections confirmed that the otc apo-ACP was quantitatively converted to desulfo-holo-ACP (data not shown).

Figure 6



Velocity versus substrate concentration plots for the reaction of ACPS with gra (black circles), fren (blue squares), otc (pink diamonds) and tcmHis6 (purple triangles) apo-ACP. Incubation conditions were as follows: gra ACP, 2 nM ACPS for 15 min; fren ACP, 18 nM ACPS for 10 min; otc ACP, 18 nM ACPS for 15 min; tcmHis$_6$ ACP, 18 nM ACPS for 15 min.

Table 3

Mass spectral data demonstrating the modification of ACPs with CoA analogs catalyzed by *E. coli* ACPS.

Substrate	CoA analog	Molecular mass (Da)	
		Calculated	Observed
E. coli ACP	none (apo)	8508	8513
	CoA	8847	8850
	homocysteamine-CoA	8861	8867
	acetonyldethio-CoA	8871	8906
	desulfo-CoA	8815	8828
otc ACP	desulfo-CoA	10224	10236

Discussion

Study of the type II aromatic PKSs *in vitro* requires sufficient quantities of active holo-ACP, that has been correctly posttranslationally modified by the covalent attachment of the 4′-phosphopantetheine cofactor on which the growing polyketide is then assembled. Overproduction of various type II PKS ACPs in *E. coli*, however, has yielded ACP exclusively or predominantly in the inactive, unmodified apo-form. Shen *et al.* [5] reported that overexpression of the tcm ACP in *E. coli* gives no holo-ACP as detected by electrospray mass spectrometry, ^{31}P NMR and [3–^{3}H] β-alanine labeling when cultures are induced in the exponential phase of growth; induction in the stationary phase of growth allows the recovery of a small percentage of tcm holo-ACP. Crosby *et al.* [6] examined the apo to holo ratio for gra, fren, otc and actinorhodin (act) ACP overproduced for 2–3 h in *E. coli*; about 30% and 2% of the gra and act ACPs respectively could be found in the holo-form, while fren and otc ACP remained unmodified. For act ACP, increasing the post-induction period to 12 h allowed 80–90% of the act ACP to be obtained in the holo-form, but these fermentation conditions also led to a significant drop in the total amount of recoverable protein [6]. Thus, standard overproduction approaches have not previously led to useful quantities of posttranslationally-modified, active holo-ACP species. Given the low recovery of PKS holo-ACPs and the implied low-level recognition of these substrates by *E. coli* ACPS, we wished to determine if *in vitro* incubation of these PKS ACPs with pure *E. coli* ACPS could be used for the preparation of functional PKS holo-ACP. Indeed, as shown by the data presented here, ACPS does catalyze transfer of phosphopantetheine to gra, fren, otc and tcm ACP to give near quantitative conversion of these proteins from their apo- to holo-forms.

The PKS ACPs examined here are also of interest for delineation of the substrate specificity of the *E. coli* holo-ACP synthase. ACPS will catalyze *in vitro* phosphopantetheinyl transfer to D-alanyl carrier protein (9 kDa) from *Lactobacillus casei* involved in the D-alanylation of lipoteichoic acid [10] and also to NodF from *Rhizobium leguminosarum* (9.9 kDa) involved in the biosynthesis of a lipo-chitin oligosaccharide molecule (R.H.L., A.M.G., C.J.W., T. Ritsema and H.P. Spaink, unpublished observations). But, ACPS will not transfer phosphopantetheine to PCP (13 kDa), a peptidyl carrier protein domain derived from the type I non-ribosomal

Figure 7

Chemical structures of the coenzyme A analogs used in this study.

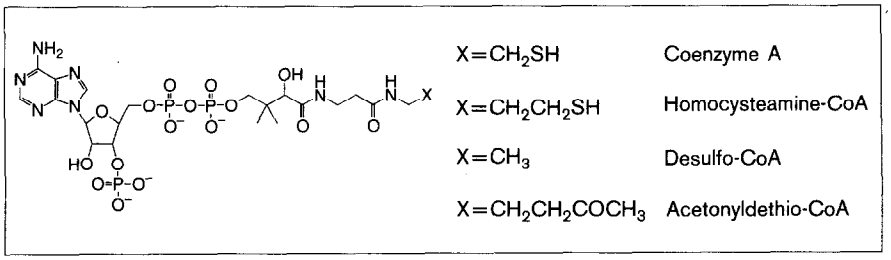
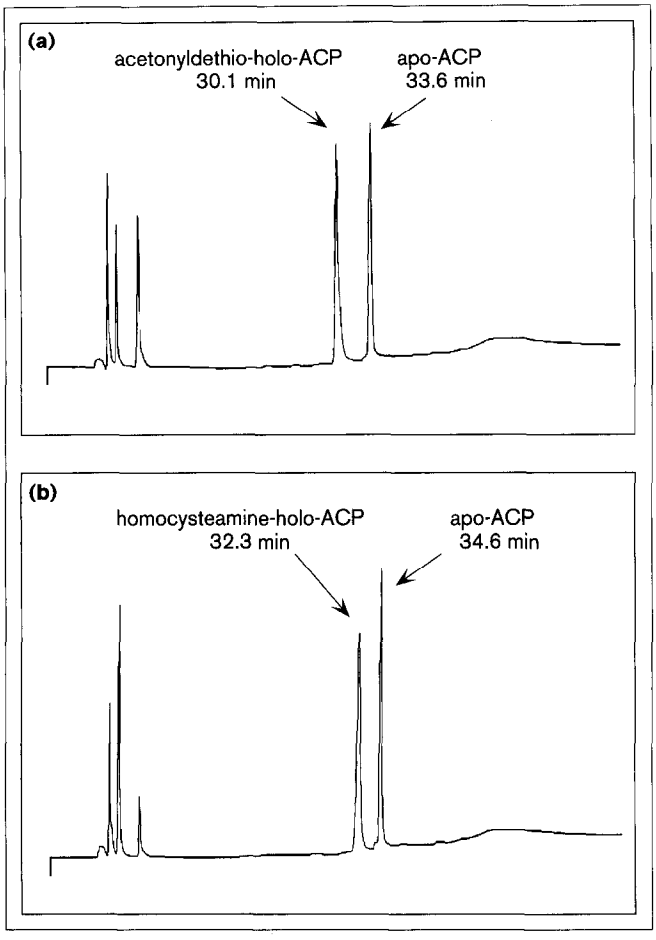


Figure 8



Modification of *E. coli* ACP with CoA analogs catalyzed by ACPS (220 nm, AUFS = 1.0). **(a)** Apo-ACP (500 μM) was incubated with 125 μM acetonyldethio-CoA in the presence and absence of 1.5 μM ACPS. Equal volumes (150 μl) of the two assays were then mixed and 200 μl of this mixture was injected onto the HPLC C18 reversed-phase column; R_t (apo-ACP) 33.6 min, (acetonyldethio-holo-ACP) 30.1 min. Single injections (data not shown) confirmed that the apo-ACP was quantitatively converted to the modified holo-form. **(b)** Sample was prepared as described above except apo-ACP was incubated with homocysteamine-CoA; R_t (apo-ACP) 34.6 min, (homocysteamine-ACP) 32.3 min. Single injections confirmed that the apo-ACP was quantitatively converted to homocysteamine-holo-ACP (data not shown).

peptide tyrocidine A synthetase responsible for tyrocidine production in *Bacillus brevis*. This dichotomy has led to our detection of a second class of phosphopantetheinyl transferases that recognize the apo-ACP domains in the multidomain peptide synthetases [11].

It is, therefore, of considerable interest to determine the effectiveness of ACPS as a phosphopantetheinylating agent for the ACPs involved in polyketide biosynthesis. The gra, fren, otc, and tcm ACPs have 19.5–23.4% similarity to the *E. coli* FAS ACP. The kinetic studies presented here indicate that, despite the fact that they all have approximately the same degree of similarity to *E. coli* ACP, there is a 25-fold range in the catalytic efficiencies of these PKS ACPs as substrates for ACPS, in the order *E. coli* ACP>gra ACP>fren ACP>tcmHis_6, otc ACP, that correlates with the degree of overall negative charge of these proteins (Table 2). The more negatively-charged an ACP substrate is, the more efficient a substrate this protein appears to be for ACPS. This hypothesis is supported by kinetic data determined for the reaction of ACPS with NodF; with an overall charge at pH7 of −10.07, NodF gives a k_{cat}/K_m value of $0.05\,\mu M^{-1}\,min^{-1}$ (A.M.G., C.T.W., T. Ritsema and H.P. Spaink, unpublished observations). The tyrocidine PCP substrate, with its essentially neutral charge, is not a substrate for ACPS [11,12], but as it is a 115 amino acid (aa) domain dissected from a 1087 aa type I nonribosomal peptide synthetase, structural factors may contribute to its inability to serve as a substrate. An increase in negative charge also correlates with an increased ability of an apo-ACP substrate to inhibit ACPS; both *E. coli* apo-ACP and gra apo-ACP are severe substrate inhibitors of ACPS, while fren apo-ACP is a weaker inhibitor and tcmHis_6 and otc apo-ACP do not inhibit ACPS. Given the complications of kinetic analysis when substrate inhibition is a factor, tcmHis_6 and otc ACP may prove more tractable substrates for further examination of the mechanism of holo-ACP synthase.

Genes encoding phosphopantetheinyl transferase(s) in *Streptomyces* spp. have not yet been identified. A crude extract from *S. coelicolor* can catalyze incorporation of phosphopantetheine into the *E. coli* ACP, but the protein(s) responsible have not been purified (A.M.G., Carreras, C., Khosla, C. and C.T.W., unpublished observations). It is not yet clear whether *Streptomyces* spp. will possess a universal phosphopantetheinyl transferase or multiple phosphopantetheinyl transferases. Multiple ACPs have been identified in *S. coelicolor* and *S. glaucescens*. In *S. glaucescens*, a constitutive ACP presumably involved in fatty acid biosynthesis is encoded by the *fab*C gene while the *tcm*M gene encodes the tetracenomycin biosynthesis ACP [3]. In *S. coelicolor* A3(2), a constitutive fatty acid ACP encoded by the *acp*P gene has also been identified which, with the ACP genes found in the *act* (actinorhodin biosynthesis) and *whi*E (spore pigment biosynthesis) PKS gene clusters, brings the number of different ACPs in this organism to three [4]. The *S. coelicolor* FAS ACP gene was unable to replace the *act* ACP gene for the production of actinorhodin *in vivo* [4], indicating the functional separation of the FAS and PKS pathways. The malonyl-coenzyme A:ACP acyltransferase in *S. glaucescens* [3] and *S. coelicolor* A3(2) [13], however, appears to function in both fatty acid and polyketide biosynthesis. It will be interesting to determine if activation of ACPs by phosphopantetheinylation represents another point of divergence and/or specificity between the FAS and PKS pathways. In *E. coli*, two and possibly three phosphopantetheinyl transferases have

been identified (ACPS, EntD, o195) each with clear specificity for their cognate substrates [11].

Significance

The feasibility of generating holo-ACPs with altered phosphopantetheine moieties has been demonstrated in this study by testing several coenzyme A analogs as substrates for ACPS. Since CoA analogs modified in the pantetheine arm are readily available, it should now be possible to routinely generate preparative quantities of holo-ACPs with phosphopantetheine groups of altered composition, length and reactivity. This opens the door to studies of the role of phosphopantetheine in the subsequent reactions of acyltransfer, condensation, reduction, dehydration and cyclization in fatty acid and polyketide biosynthesis. For example, the desulfopantetheinyl and the acetonyldethiopantetheinyl holo-ACP forms may be selective inhibitors of particular steps in polyketide elongation. Similarly, in lieu of an as yet uncharacterized *Streptomyces* phosphopantetheinyl transferase, the ability of ACPS to modify the PKS ACPs studied here makes it a valuable reagent for the large-scale preparation of active PKS holo-ACP for further enzymatic studies. Also, coexpression of streptomycete apo-ACP molecules in an *E. coli* strain overproducing the *E. coli* holo-ACP synthase should yield functional holo-ACP and species competent for aromatic polyketide assembly.

Materials and methods

Overproduction and purification of Streptomyces *apo-ACPs*

Plasmids containing genes for the following *Streptomyces* PKS acyl carrier proteins were kindly provided by Chaitan Khosla, Stanford University: granaticin ACP (*S. violaceoruber*), frenolicin ACP (*S. roseofulvus*), oxytetracycline ACP (*S. rimosus*) and tetracenomycin ACP (*S. glaucescens*). These plasmids were used as the templates for PCR amplification of these genes using the following primers: gra ACP forward primer: 5′-TTCCAGCTAGGATC*ATATG*GCTCGTCTGACCCTGGACGGTCTGCG-3′, *gra* ACP reverse primer: 5′-GATTAGTCA*CAAGCTTT*CAGGCCGCCTCGGCCTGGGC-3′; *fren* ACP forward primer: 5′-ACATTCAGGATAC*CATATG*AGCGCACTGACCGTTGACGAC-3′, fren ACP reverse primer: 5′-AATGGCGAATAAGCTTTCAGGCGGTGGCCGGGGTGGTGTT-3′; *otc* ACP forward primer 5′-GATCTCATGCACA*CATATG*ACCCTGCTGACCCTGTCCGA-3′, otc ACP reverse primer 5′-AACTCGATGC*AAGCTTT*CACTTGTCCCGCGCGGCGCCA-3′; *tcm* ACP forward primer 5′-AAGTGCGATGCGA*CATATG*CCGCAGATCGGCCTGCCGCGTCTCGTC-3′, tcm ACP reverse primer: 5′-GTAGCTTAGC*AAGCTTT*CAGGCGACCTCCCCGGCCGTCTC-3′ (all primers from Integrated DNA Technologies). The forward primers introduce an *Nde*I restriction site (italic) while the reverse primers introduce a *Hin*dIII restriction site (italic). In addition, the forward primers optimized the codon usage for the first eight codons of the ACP genes to facilitate expression in *E. coli*. The *Nde*I/*Hin*dIII-digested PCR product was cloned into pET22b (Novagen) and transformed into competent DH5a and BL21(DE3) *E. coli* cells. The identity of the recombinant ACP genes was confirmed by DNA sequencing (Biopolymer Laboratory, Department of Biological Chemistry and Molecular Pharmacology, Harvard Medical School). Surprisingly, DNA sequencing revealed that the *tcm* ACP gene had inadvertently been obtained with a carboxy-terminal His_6 tag supplied by the pET22b vector and appending the amino acid sequence LEHHHHHH to the overexpressed tcm ACP.

Cultures of BL21(DE3)pET22b-gra, BL21(DE3)pET22b-fren, BL21(DE3)pET22b-otc, and BL21(DE3)pET22b-tcmhis_6 (2×1l, 2×YT media) were each grown at 37°C to an optical density (O.D.) of 0.5–0.8 and then induced with 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG). Growth continued for 3–4 h and cells were harvested by centrifugation. The overproduced ACPs were released and purified by a variation of the freeze/thaw method of Morris *et al.* [14]. The harvested cells were frozen as a cell pellet at −80°C for 24 h and then thawed in buffer A (50 mM Tris·HCl, 2 mM dithiothreitol (DTT), 2 mM EDTA, pH 7.5) to release the overexpressed ACPs without lysing the cells. This freeze/thaw supernatant was then loaded onto a Q-Sepharose column (2.5×10 cm) which had previously been equilibrated with buffer A. For purification of gra ACP, a 200 ml gradient of 0–0.4 M KCl (in buffer A) followed by 400 ml of a gradient from 0.4–0.8 M KCl was then applied at a flow rate of 2 ml min^{-1} with ACP eluting at ~0.4 M salt as indicated by 20% native PAGE of the column fractions. For purification of fren ACP, 150 ml of a 0–0.3 M KCl gradient (in buffer A) was followed by a 300 ml 0.3–0.6 M KCl gradient and a 150 ml 0.6–0.8 M KCl gradient with ACP again eluting at ~0.4 M salt. For otc ACP purification, the column was washed with buffer A (100 ml) and gradients of 0–0.2 M KCl (150 ml), 0.2–0.5 M KCl (300 ml) and 0.5–0.8 M KCl (50 ml) were applied (ACP elutes at ~0.28 M KCl). Finally, for tcmHis_6 ACP purification, the column was washed with 50 ml buffer A and gradients of 0–0.25 M KCl (150 ml), 0.25–0.55 M KCl (300 ml) and 0.55–0.8 M KCl (100 ml) applied (ACP elutes at ~0.35 M KCl). Fractions containing each ACP as judged by 20% native PAGE were pooled and dialyzed against 2 l buffer A. At this stage in the purification, a sample of each ACP was submitted for amino-terminal protein sequencing (10 cycles) and MALDI-TOF mass spectral analysis (Howard Hughes Medical Institute Biopolymer Facility, Harvard Medical School).

To obtain samples of each ACP purified to near homogeneity, the BioCAD™ SPRINT™ perfusion chromatography system (PerSeptive Biosystems, Inc.) was used. A portion of the dialyzed ACP samples following Q-sepharose chromatography was applied to a POROS 20HQ anion-exchange column at a flow rate of 10 ml min^{-1}. The optimum pH for separating the ACPs from the remaining contaminants was determined to be gra ACP, pH 9.0; fren ACP, pH 6.0; otc ACP, pH 9.0; and tcmHis_6 ACP, pH 9.0. Proteins were eluted with a gradient of 0–0.5 M NaCl in 10 mM Tris, 10 mM Bis-tris propane of the indicated pH. The tcmHis_6 ACP sample was additionally purified by nickel chelate chromatography according to the manufacturer's instructions (Novagen). The buffer was exchanged for 50 mM Tris·HCl, pH 8.0 and the protein samples concentrated using Centriprep 3 (Amicon). Protein concentration was determined by measuring the absorbance at 280 nm using the following calculated extinction coefficients [15]: gra ACP 1400 $M^{-1}cm^{-1}$, fren ACP 2560 $M^{-1}cm^{-1}$, otc ACP 2680 $M^{-1}cm^{-1}$, and tcmHis_6 ACP 2680 $M^{-1}cm^{-1}$.

Preparation of the CoASH analogs

Desulfocoenzyme A was purchased from Sigma Chemical Company. Acetonyldethio-coenzyme A was prepared as described previously [16]. Homocysteamine-coenzyme A was prepared as follows. A suspension of 0.88 g homocysteamine hydrochloride [17,18] in 1 ml H_2O was added to 10 mg adenosine 5′-(trihydrogen diphosphate) 3′-(dihydrogen phosphate) 5′-[(R)-3-hydroxy-4-[[3-[propylthio]-3-oxopropyl]amino]-2,2-dimethyl-4-oxobutyl] ester [19] in 1 ml H_2O and the pH adjusted to 10 with 6 M NaOH. The reaction was stirred at room temperature while nitrogen was gently bubbled through the solution. The reaction was monitored by analytical HPLC and complete disappearance of the starting CoA synthon (R_t = 17.3 min) and appearance of product (R_t = 13.7 min) was observed in 4 h. The reaction was filtered, the pH was adjusted to 4.5, and the product partially purified by preparative HPLC (5 min 5% methanol (solvent B) followed by a linear gradient to 45% B over 40 min, product eluted at 25–32 min). The product was further purified on a DEAE-Sepharose column (20×2.5 cm, 0–0.2 M NaCl pH 2.8, product eluted at 0.13 M NaCl) to yield 4.5 mg homocysteamine-CoA. λ_{max} = 260 nm. ^{1}H NMR (as potassium

salt, 400 mHz, D_2O): δ 0.724 (s, 3H), 0.852 (s, 3H), 1.65–1.72 (m, 2H), 2.392 (t, 2H, *J*=6.4 Hz), 2.447 (t, 2H, *J*=7.0 Hz), 3.174 (t, 2H, *J*=6.8 Hz), 3.409 (t, 2H, *J*=6.2 Hz), 3.516 (dd, 1H, *J*=4.8, 10.0 Hz), 3.785 (dd, 1H, *J*=4.8, 10.0 Hz), 3.962 (s, 1H), 4.195 (br s, 2H), 4.551 (br s, 1H), 6.160 (d, 1H, *J*=6.0 Hz), 8.306 (s, 1H), 8.561 (s, 1H). Mass Spec HRMS (FAB): $(M-H+)^-$ calc'd for $C_{22}H_{37}N_7O_{16}P_3S$ *m/z* 780.191, found 780.126.

Analytical and preparative-scale HPLC experiments were performed using a Perkin-Elmer 250 HPLC with a PE LC-235 diode array detector and a gradient of methanol (solvent B) in aqueous potassium phosphate (solvent A, 50 mM KH_2PO_4 for analytical runs, 10 mM KH_2PO_4 for preparative runs). Analytical HPLC was done on a Rainin Microsorb C18 column (4.6 mm × 25 cm) with monitoring at 215 and 260 nm. Compounds were eluted with a flow rate of 1 ml min^{-1} with 5% solvent B for 2 min, followed by a linear gradient to 60% solvent B over 12 min, and then maintained at 60% solvent B. Preparative scale HPLC was done on a Rainin Microsorb C18 column (21.4 cm × 25 cm) with monitoring at 215 and 280 nm and a flow rate of 10 ml min^{-1}. Mass spectral analysis was performed at the University of California at Riverside Mass Spectrometry Facility, Riverside, CA. The concentrations of CoA analog solutions were determined using $\varepsilon_{260} = 15\,400\ M^{-1}cm^{-1}$.

Assay for apo-ACP to holo-ACP conversion by transfer of ^{3}H-phosphopantetheine from ^{3}H-coenzyme A

The radioassay for determination of phosphopantetheinyl transferase activity has been previously described [7,9,11]. Briefly, in a final volume of 100 μl, substrate (apo-gra, fren , otc or tcmHis$_6$ ACP) was incubated with 75 mM Tris·HCl, pH 8.8, 10 mM $MgCl_2$, 25 mM DTT, 220 μM [^{3}H]-(pantetheinyl)-CoASH (specific activity 105 μCi μmol^{-1}, 70% label in phosphopantetheine portion of the molecule) and *E. coli* holo-ACP synthase (prepared as described in [7]) at an appropriate concentration at 37°C for a specified time. Reactions were quenched with 800 μl 10% trichloroacetic acid (TCA), bovine serum albumin (375 μg) was added as a carrier, and protein precipitate pelleted by centrifugation. This pellet was washed 3× with 10% TCA and the pellet was dissolved in 1 M Tris base. The amount of radiolabel incorporated into the solubilized protein pellet was then determined by liquid scintillation counting. For the K_m measurements of the *Streptomyces* ACPs as substrates for holo-ACP synthase, the following conditions were used: gra ACP was incubated at varying concentrations over the range 5–60 μM with 2 nM ACPS for 15 min; fren ACP (range 5–80 μM) was incubated with 18 nM ACPS for 10 min; otc ACP (range 5–180 μM) was incubated with 18 nM ACPS for 15 min; tcmHis$_6$ ACP (5–100 μM) was incubated with 18 nM ACPS for 15 min. The amount of tritium incorporation at each ACP concentration was determined in triplicate. Samples of each *Streptomyces* ACP were submitted for MALDI-TOF mass spectral analysis following incubation for several hours with 1.8 μM ACPS using the same conditions described for the radioassay except that 2 mM CoASH or 200 μM desulfoCoA was used as indicated.

HPLC and mass spectrometry assay for modification of ACPs

In a 1.5-ml microcentrifuge tube, 1 mM CoA (or 125 μM CoA analog), 500 μM apo-ACP (or 50 μM otc apo-ACP), 20 mM $MgCl_2$, 10 mM DTT, 150 mM Tris·HCl, pH 8.8, and 1.5 μM ACPS (100 μl final volume) were incubated at 37°C for 30 min. Reactions for HPLC analysis were quenched with 900 μl 0.1% trifluoroacetic acid (TFA). A sample (200 μl) of this solution was injected onto a C18 reversed phase analytical column (Vydac) which had been equilibrated with 15% isopropanol in 0.1% TFA (eluant A) at 0.5 ml min^{-1}. Absorbance at 220 nm was monitored. The sample was eluted from the column with 2.5 ml 100% A, a 10 ml linear gradient to 40% B (75% isopropanol in 0.1% TFA), followed by a 7.5-ml linear gradient to 100% B at a constant flow rate of 0.5 ml min^{-1}. Under these conditions holo-ACP elutes before apo-ACP. Samples for MALDI-TOF mass spectrometry analysis were submitted as the crude incubation mixtures (Biopolymers Facility, Howard Hughes Medical Institute, Harvard Medical School).

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