The Unmodified (Apo) Form of Escherichia coli Acyl Carrier Protein Is a Potent Inhibitor of Cell Growth*

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Acyl carrier protein (ACP) is the carrier of fatty acids during their synthesis and utilization. ACPs (or ACP-like protein domains) have been found throughout biology and share significant amino acid sequence similarities. All ACPs undergo a post-translational modification in which 4-phosphopantetheine is transferred from CoA to a specific serine of apo-ACP. This modification is essential for activity because fatty acids are bound in thioester linkage to the sulphydryl of the prosthetic group. Overproduction of Escherichia coli ACP from multicyclop transplasmds strongly inhibits growth of E. coli. We report that upon overexpression of ACP in E. coli post-translational modification is inefficient and the apo-protein accumulates and blocks cell growth by inhibition of lipid metabolism. Moreover, a mutant form of ACP that is unable to undergo post-translational modification is a potent inhibitor of growth. Finally, we observed that an increase in the efficiency of modification of overexpressed ACP results in decreased toxicity. The accumulated apo-ACP acts as a potent inhibitor of growth. The degree of inhibition depended upon the species of donor acyl chain. Utilization of cis-vaccenoyl-ACP by the sn-glycerol-3-phosphate acyltransferase was inhibited to a much greater extent by apo-ACP than was utilization of palmitoyl-ACP. 1-Acetyl glycerol-3-phosphate acyltransferase was also inhibited in vitro by apo-ACP, although not at physiologically relevant concentrations. These in vitro data are supported by in vivo labeling data, which showed a large decrease in cis-vaccenate incorporation into phospholipid during overproduction of ACP, but no decrease in the rate of synthesis of long chain acyl-ACPs. These data indicate that acylation of sn-glycerol 3-phosphate is the major site of inhibition by apo-ACP.

Fatty acid synthesis in Escherichia coli is the prototypical type II fatty acid system in which each cycle of condensation, reduction, dehydration, and further reduction are catalyzed by a group of monofunctional polypeptides (for a review, see Magnuson et al. (1993)). The growing fatty acid moves among these enzymes not as a free carboxylic acid, but in a form covalently linked to a protein called acyl carrier protein (ACP). Upon completion of acyl chain synthesis, ACP serves as the carrier of the mature fatty acid during subsequent transfer to sn-glycerol 3-phosphate (G3P) and lipid A. The presence of the ACP esterified to fatty acids allows for recognition by the enzymes involved in fatty acid synthesis and prevents degradation of the newly synthesized fatty acid (fatty acids are catabolized only as CoA thioesters).

ACP has been found to play an essential part in a growing number of processes outside of fatty acid biosynthesis. In Rhizobia, a specialized ACP is required in the acylation of oligosaccharides required for nodulation (Spanik et al., 1991). In Streptomycetes, either an ACP or an ACP-like domain is required for the synthesis of polyketide antibiotics (Hopwood and Sherman, 1990). ACP or ACP-like domains have also been found to act as carriers of activated amino acids during synthesis of non-ribosomally synthesized antibiotics (Lipman, 1980) and lipoteichoic acids (Heaton and Neuhaus, 1993). In E. coli, ACP has been found in vitro to be essential for the transglucosylation reaction required in the synthesis of membrane-derived oligosaccharides (Therisod and Kennedy, 1987). However, the synthesis of these oligosaccharides seems to be distinct from the reactions mentioned above in that apo-ACP is active as a cofactor for this enzyme.

E. coli ACP is a low molecular mass (8,860 Da) (Magnuson et al., 1993), acidic (pI 4.1) (Vandenboom and Cronan, 1989), very abundant (≈6 × 10⁴ molecules/cell) cytoplasmic protein. Although the crystal structure has not been determined, NMR studies have indicated that ACP exists in solution as a rod shaped protein made up of four α helices that interact to form a hydrophobic sheet capable of accommodating the prosthetic group and up to 6 carbons of the growing fatty acid chain. The structure of the molecule is stabilized upon acylation, most likely through interactions between the fatty acyl chain and hydrophobic faces of the helices (Magnuson et al., 1993).

ACP is synthesized as an apo protein, which undergoes post-translational modification by the addition of a 4'-phosphopantetheine, to serine 36 of the amino acid backbone (for a review, see Magnuson et al. (1993)). This post-translational modification is catalyzed by holo-ACP synthase. This enzyme uses CoA as the 4'-phosphopantetheine donor and specifically modifies serine 36 of ACP. The growing fatty acid chain is attached to the terminal sulfhydryl group of the phosphopantetheine, the only sulfhydryl group of E. coli ACP. All known ACP (or ACP-like domains) undergo this modification and all share sequence similarity around the modified serine (Vanden Boom and Cronan, 1989). Mutants impaired in the ability to post-translationally modify ACP remain viable de
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Despite having only one third of their ACP present in the functional form, suggesting that ACP is present in E. coli in functional excess (Vanden Boom and Cronan, 1989).

The prosthetic group of ACP is turned over rapidly in vivo as a result of the action of the enzyme ACP phosphodiesterase. This enzyme catalyzes the removal of the prosthetic group from ACP, resulting in free 4'-phosphopantetheine and apo-ACP. The activity of this enzyme appears to be stimulated by decreasing CoA levels (Magnuson et al., 1993), but little is known about the role of ACP phosphodiesterase in normal E. coli physiology. The gene encoding this enzyme has not been cloned, and no mutants exist (Magnuson et al., 1993). Halo-ACP synthase and ACP phosphodiesterase constitute a futile cycle, and previous workers (Elovson and Vagelos, 1975) have suggested that the action of this cycle would be a likely point of regulation. However, later experiments have not supported this prediction (J.ackowski and Rock, 1983).

Cloning of the gene (acpP) encoding ACP was long precluded due to the inability to stably maintain the gene in high copy number plasmids (Vanden Boom et al., 1989). This instability was first recognized through use of a synthetic gene encoding ACP to be the direct result of overexpression of the gene product. This result was unexpected in that ACP is a very abundant and extremely soluble protein. Moreover, ACPs from other organisms have been overproduced in E. coli (albeit at lower levels) without the associated toxicity (Guerra et al., 1988). We have examined the cause of this toxicity and have determined that overexpressed ACP is incompletely post-translationally modified and that the unmodified apo-ACP is a powerful and specific inhibitor of lipid metabolism.

**EXPERIMENTAL PROCEDURES**

**Bacterial Strains—** All strains used in this work are derivatives of E. coli K-12. Strain MP3 (pban6 Fad E sbx 1 (bio-gal)) (Polacco and Cronan, 1981) was transformed with both pMR19 and pMS421 to yield strain DK552. Plasmid pMR19 contains a synthetic version of the acpP gene under control of the tac promoter (Rawlings, 1993) and was derived from plasmid pK223-3 (Broius and Hdy, 1984). Plasmid pMS421 (Grana et al., 1988) contains the lac d’ mutation and was used to regulate transcription of the acpP gene. Strain MP4 (pban6 Fad E sbx 1 (bio-gal)) (Polacco and Cronan, 1981) was transformed with plasmids pMR19 and pMS421 to yield strain DK554. Strain DV79 (met81 pban2 corl6 friz 10) (Vallari and Jakkowski, 1988) was transformed with pMS421 and pMR19 to yield strain DK739. Strain SJ 16 (metB1 relA1 spoT1 L- gyrA216 panD2 zed-2200 Tn 10 F-) (J.ackowski and Rock, 1981) was transformed with two plasmids (pMS421 and pMR19) to give strain DK574. Strain MP3 was transformed with pHCl12 to give strain DK75, or pHCl12 and pMR24 to give strain DK75B. Plasmid pHCl12 encodes a mutant E. coli thioesterase I lacking the leader peptide under control of the arabinose promoter (Cho and Cronan, 1995). Plasmid pMR24 contains the wild type acpP gene expressed from the native promoter (Rawlings and Cronan, 1992).

**DNA Manipulations—** Plasmid pDK655 was constructed by ligating the acpP-containing EcoRI-HindII fragment of pMR19 into pTZ19U (Mead et al., 1986) as described previously (Rawlings and Cronan, 1992). Site-directed mutagenesis was performed as described previously (Kunkel, 1987). Single-stranded uracil-containing DNA was used as a template for synthesis using mixed mismatch-containing oligonucleotide TGGGTGCTGACA (GCTTCTGACAC (Genetic Engineering Center University of Illinois Urbana-Champaign) and the Mutate-Gene site-directed mutagenesis kit (Bio-Rad). The resulting double-stranded transformants were sequenced by the double-stranded chain termination technique (Tabor and Richardson, 1987, 1989) using Sequenase 2.0 and plasmid DNA. Plasmids pMS421 (control) or pMR19 (mutant) were then digested with EcoRI and HindIII, and the resulting acpP-containing fragments were ligated to EcoRI-HindII-digested pK223-3 to give plasmids pDK675 and pDK685.

**Media—** Culture media were used as described previously (Davis et al., 1982) or minimal salts medium E (Davis et al., 1980) supplemented with 0.4% glucose and 0.1% vitamin-free casein hydrolysate (Difco). Spectinomycin (30 µg/ml), ampicillin (100 µg/ml), kanamycin (40 µg/ml), tetracycline (10 µg/ml), pantothenate (120 µg/ml), or β-alanine (5 µg/ml) were added as required. Solid media contained 1.5% agar. All cultures were grown at 37 °C. Growth of liquid cultures were monitored with a Klett-Summner colorimeter equipped with a green filter.

Analysis of ACP Pools—Cells were grown in E media containing glucose and vitamin-free casein hydrolysate to early log phase (3 × 107 cells/ml). IPTG (1 mM) was then added, and at various time points Trasnorm (ICN Biochemical, 50 µCi) was added to 1 ml cell culture. The culture was allowed to shake for 10 min at 27 °C, followed by addition of 10% trichloroacetic acid directly to the shaking culture, and incubation on ice for 30 min. The trichloroacetic acid-treated culture was then centrifuged for 10 min at 16,000 × g, resuspended in 50 ml MES, 10 mM N-ethylmaleimide (pH 6.1) (Post-Bettenmill et al., 1991) and either analyzed by urea-PAGE (see below) or quick-frozen in a dry ice-acetone bath and stored at −70 °C. ACP species were analyzed as described previously (Post-Bettenmill et al., 1991). Briefly, extracts were prepared as described above; urea was added to 2.5 M, followed by addition of sample buffer to a total volume of 50 µl. Samples were then fractionated on a 13% PAGE gel containing 2.5 M urea for approximately 2.5 h at 15 °C. The gels were then fixed, dried and exposed to x-ray film. Apo-ACP standards were prepared by in vitro transcription/translation of pMR19 using the S30 in vitro transcription/translation kit (Promega). The extract plus plasmid DNA was assembled according to manufacturer’s instructions and incubated for 1 h at 37 °C, followed by addition of trichloroacetic acid to 2.5% and incubation on ice for 30 min. The precipitate was then centrifuged at 16,000 × g, resuspended in resuspension buffer (see above), quick-frozen in a dry ice-acetone bath, and stored at −70 °C. Labelled mutant ACP with [3-3H]lalanine was done as follows. Strains harboring plasmids which contained the wild type and mutant acpP genes were grown to early log phase in the presence of 10 µCi [3-3H]lalanine (92.6 Ci/mmol) followed by addition of 1 µCi IPTG and growth for 6 h. The labeled cell was then centrifuged for 10 min at 16,000 × g, washed twice with resuspension buffer (see above), trichloroacetic acid was added to 2.5% and the samples were incubated on ice for 10 min. The precipitates were then collected by centrifugation at 16,000 × g and the resulting pellet washed three times with 1% trichloroacetic acid followed by scintillation counting.

**Lipid Analysis—** Labeling of fatty acids with [14C]acetate was done as described previously (Geiman and Cronan, 1972). Briefly, strains were grown to early log phase in minimal E media, followed by addition of sodium [1-14C]acetate (5 µCi). The strain was then cultured for 10 min at 37 °C and the labeling stopped by addition of 6 ml of 2 M methanol: chloroform and unlabeled carrier cells. The lipids were then extracted by the method of Bligh and Dyer (1959), transesterified to form methyl esters, and fractionated by argentation thin layer chromatography (Morris et al., 1967). Determination of incorporation of acetate into free fatty acid pool in strains overproducing the leaderless teA was accomplished by Bligh and Dyer extraction and fractionation on Silica Gel G as described previously (Jiang and Cronan, 1994). Labeling with [1H]palmitate followed an identical protocol except that labeling was done with 5 µCi of [9,10-3H]palmitic acid.

G3P Acyltransferase Assay—G3P acyltransferase activity was assayed as described previously (Rock et al., 1981). Briefly, 0.1 mM Tris-HCl (pH 8.5), 1 mg/ml bovine serum albumin, 12.5 µM acylACP, 5 mM MgCl2, 250 µM [1,3-14C]G3P (10,000 cpm/nmol), and 10 µg of E. coli membrane protein extract were incubated in the presence of varying amounts of purified apo-ACP (see below) for 10 min at 23 °C followed by transfer to a Whatman No. 3MM disk. Disks were then washed with 20 ml of 5% trichloroacetic acid, followed by 15 ml of 1% trichloroacetic acid. The disks were then impaled on straight pins, dried under a stream of hot air, and counted in 2 ml of BCS scintillation fluid in a Beckman scintillation counter. Membranes were prepared as described previously (Rock et al., 1981) with the modification that the inner and outer membranes were not separated. Acyl-ACP’s were prepared as described previously (Rock and Garwin, 1979) with the modification that acyl-ACP synthetase was purchased from Sigma.

1-Acyl-G3P Acyltransferase Assay—The assay was performed as described previously (Coleman, 1990) with the following modifications. Briefly, [3-3H]-labeled 1-palmitoyl-G3P (1000 cpm) (see below), 20 µM acylACP, 1.5 mg/ml bovine serum albumin, and 20 µg of membrane protein (Coleman, 1990) were incubated with varying amounts of apo-ACP. The reaction was started by addition of the membrane extract and incubation for 10 min and halted by addition of 0.4 ml of 2:1 methanol:chloroform. The methanol:chloroform was then removed under a stream of nitrogen, the pellet was dissolved in chloroform and spotted on a Silica Gel G plate, J. B. Ohlrogge, personal communication.
followed by development using chloroform:methanol:acetic acid:water (25:15:4:2, by volume). The plate was then dried and exposed to a Molecular Dynamics PhosphorImager for quantitation.

Preparation of Labeled 1-Acyl-G3P—Labeled 1-acyl-G3P was prepared using the ability of diglyceride kinase to phosphorylate mono-acyl glycerides (Nachiappan and Rajasekharan, 1994). Briefly 1.4 mM rac-palmitylglycerol, 0.03 mM MgCl₂, 3.9 mM [γ³²P]ATP, 29 mM sodium phosphate (pH 7.2), and 0.1% Cutscum detergent was incubated with 0.1 units of diglyceride kinase (Calbiochem) for 30 min at 37°C. The labeled 1-palmitoyl-G3P was then isolated by utilizing the charged nature of 1-palmitoyl-G3P. The 1-palmitoyl-G3P was partitioned into the methanol phase of the Bligh and Dyer extraction. The methanol phase was then acidified by adding 48 mM ammonium acetate and chloroform. The chloroform phase was then separated from the methanol phase by centrifugation, followed by washing with KCl and water twice. The chloroform was then removed by a stream of nitrogen under heat, and the resulting pellet was brought up in methanol and 0.5 mL of water. The methanol was then evaporated in the presence of heat, and the resulting labeled 1-palmitoyl-G3P was stored at −20°C. This protocol yielded radiochemically pure 1-palmitoyl-G3P.

Purification of Apo-ACP—Apo-ACP was purified as described previously (Rock and Cronan, 1980) with the following modifications. Strain DK554 was grown to 8 × 10⁸ cells/mL. IPTG (1 mM) was added and the culture incubated for another 6 h. Cells were then centrifuged at 6,000 × g and concentrated 10-fold in 50 mM Tris-HCl (pH 7.0), followed by two passages through a French pressure cell at 18,000 p.s.i. DNase (55 units) was added and RNase (100 units) was added and incubated at 37°C for 30 min. The pH of the cell lysate was increased to pH 8.5, followed by incubation for 1 h at 37°C. The cells were then diluted 10-fold in 50 mM Tris-HCl (pH 7.0) and an equal volume of isopropanol was added followed by storage overnight at 4°C. The precipitate was then centrifuged at 6,000 × g, the pH was titrated to pH 6.5, and DEAE-cellulose (DE-53, Whatman) (10 g) equilibrated to pH 6.1 was added and stirred overnight at 4°C. The DE-53 resin was then washed (in a Büchner funnel containing Whatman 1 paper) with Tris-HCl (pH 6.1) containing 0.25 M LiCl. The resin was then diluted with a column and the ACP eluted with 200 mM of 10 mM Tris-HCl (pH 6.1) containing 0.5 M LiCl. The elution fraction was then triturated with acetic acid to pH 3.9 and the ACP allowed to precipitate followed by centrifugation and dissolution of the pellet in 10 mM Tris-HCl (pH 7.0). Ammonium sulfate was added to 80%, followed by incubation on ice for 1 h. The precipitate was then removed by centrifugation at 6,000 × g and the ACP recovered by acid precipitation (as described above), followed by dialysis against 10 mM Tris-HCl (pH 7.0) overnight. This procedure yielded essentially pure apo-ACP, as judged by gel electrophoresis.

RESULTS AND DISCUSSION

Effects of ACP Overexpression on Cell Growth—The physiological effects of overproduction of ACP were studied with a plasmid that placed a synthetic version of the acpP gene under control of the inducible tac promoter. Addition of IPTG to the growth media resulted in overexpression (~20-fold) of ACP. The polypeptide produced by this synthetic gene has been shown by a number of analyses to be indistinguishable from that of the wild type acpP gene (Rawlings, 1993). The growth rate of an E. coli strain overexpressing ACP (Fig. 1) was followed by measurement of turbidity. Approximately 1 h after induction the growth rate began to decline and growth virtually ceased 3–4 h after induction. The decline in growth rate was accompanied by a marked loss of viability (~500-fold) (Rawlings, 1993). Filamentation accompanied the loss of viability, but no cell lysis occurred. We have found that even 2–5-fold overexpression of ACP results in detectable accumulation of unmodified ACP and growth inhibition, although the degree of toxicity is proportional to the level of overexpression.

Accumulation of Apo-ACP—ACP is one of the most abundant soluble proteins in E. coli and thus it seemed unlikely that higher concentrations of ACP should result in growth inhibition. Therefore we attempted to determine if ACP overproduction was accompanied by novel forms of E. coli ACP that might be the cause of the toxicity. We examined the composition of the ACP pool produced by E. coli during ACP overproduction. Cells labeled with [³⁵S]methionine were subjected to electrophoresis on 13% polyacrylamide gels in the presence of urea (Fig. 2). In this gel system, the migration of an ACP depends on post-translational modification and subsequent acylation with long chain acyl ACPs migrating more rapidly than shorter chain length fatty acyl ACPs. Surprisingly, the predominant species seen at late time points was a species that comigrated with apo-ACP. Clearly, when ACP is overproduced from a multicopy plasmid, the cellular capacity for post-translational modification of ACP becomes rate-limiting. Because no detectable pool of apo-ACP exists in wild type cells (J. Ackowski and Rock, 1983), it seemed possible that accumulation of this form of ACP could be responsible for the toxic effects of overproduction.

Overexpression of a Mutant ACPs Defective in Post-translational Modification—To more directly test the effects of apo-ACP on cell growth, we constructed mutants of ACP defective in post-translational modification. Serine 36 (the amino acid covalently linked to 4'-phosphopantetheine) was changed to either alanine or threonine by site-directed mutagenesis. The inability of the mutant ACPs to undergo efficient post-translational modification was measured by monitoring the relative incorporation of β⁹⁹⁷⁶⁸¹-Halanine into ACP. β¹⁰¹⁷¹¹-Alanine is a precursor of CoA and specifically labels the ACP prosthetic group. No detectable post-translational modification of the S36T protein was seen, whereas the S36A mutant protein seemed to inhibit modification of the chromosome-encoded wild type protein (Fig. 3B). The S36T mutant was chosen for further study because the structural similarity of threonine to serine resulted in no noticeable change in the structure of apo-ACP as judged by gel electrophoresis (the alanine-substituted ACP migrated aberrantly on these gels). Production of the S36T mutant protein was growth-inhibitory (Fig. 3A) consistent with the hypothesis that the apo form of ACP is responsible for the growth arrest.

Manipulation of the Apo-ACP Levels in ACP-overexpressing Cells—If apo-ACP accumulation is toxic, a decrease in the level of apo-ACP relative to holo-ACP should give a corresponding decrease in lethality. Since CoA is the direct donor of 4'-phosphopantetheine to apo-ACP, an increase in the intracellular CoA concentration should give a greater level of modification of overexpressed ACP. Strain DK739 contains a mutant pantethenate kinase refractory to feedback inhibition by non-esterified CoA (Vallari and J. Ackowski, 1988). Pantetheinase kinase has been shown to be the rate-limiting step for CoA biosynthe-
the decrease in differential inhibition of acetate incorporation into the three major acetate incorporation into lipids. More striking was the difference of the expression. Labeling carried out at various times following induction. ACP-ACP dimers formed by disulfide cross-linking during sample preparation) decreased approximately 6-fold when ACP was overproduced in the feedback-resistant strain grown with high pantothenate. It should also be noted that the strain containing the wild type pantothenate kinase showed a similar tendency toward reduced relative apo-ACP pool size when grown with high pantothenate, although the effect was less pronounced than in the feedback-resistant strain. Apo-ACP Is an Inhibitor of Lipid Biosynthesis—Since the sole known essential function for ACP in E. coli is its role in lipid metabolism, it seemed likely that the effects of apo-ACP resulted from inhibition of some aspect of lipid metabolism. We, therefore, measured the rate of incorporation of labeled acetate (a precursor of all E. coli lipids) into lipids during ACP overexpression. Labeling carried out at various times following induction of the acpP gene showed a pronounced decrease (Fig. 5) in acetate incorporation into lipids. More striking was the differential inhibition of acetate incorporation into the three major fatty acid species found in E. coli phospholipids. The most drastic and immediate decrease was in cis-vaccenate observed within 40 min following acpP induction. It should be noted that the decrease in cis-vaccenate occurred before any change in

![Fraction of ACP species produced during ACP overexpression](Image 2)

**Fig. 2.** Fractionation of ACP species produced during ACP overexpression. E. coli strain DK552 was cultured to early log phase followed by addition of 1 mM IPTG as described under “Experimental Procedures.” One-ml samples were removed at the time points listed below, cultured in the presence of 50 μCi of Trans^35S-label (a mixture of labeled methionine and cysteine), and fractionated on 13% PAGE containing 2.5 M urea. Odd-numbered lanes were labeled at 0, 20, 40, 60, 120, and 240 min after addition of IPTG, respectively. Even-numbered lanes were labeled at the same time points, but in the absence of IPTG.

![Effects of overexpression of mutant ACPs](Image 3)

**Fig. 3.** Effects of overexpression of mutant ACPs. Panel A, inhibition of growth rate by overexpressed mutant ACP. Strains harboring pDK676 (wild type) and pDK685 (S36T) were cultured to early log phase and IPTG was added. [ ], pDK676 without induction; [ ], pDK676 with induction; [ ], pDK685 with induction. Panel B, β-[^3H]alanine labeling of wild type and mutant ACPs. Strains containing pDK675 (encoding wild type ACP), pDK675 (mutant S36A ACP), or pDK685 (mutant S36T ACP) were grown to early log phase in the presence of β-[^3H]alanine, followed by addition of IPTG. The incorporation of label into trichloroacetic acid-precipitable material was then measured by scintillation counting as described under “Experimental Procedures.” β-Alanine is a precursor of CoA and specifically labels the ACP prosthetic group. The plasmids present in each strain are denoted at the bottom of the figure. Lane 1, S[16]DK675 (lane 2, S[16]DK676; lane 3, S[16]DK676; lane 4, S[16]DK676)

Apo-ACP Fails to Inhibit Synthesis of Long Chain Fatty Acids—The observed decrease in incorporation of labeled acetate into lipids (Fig. 5) could be due to inhibition of the synthesis of long chain fatty acyl ACPs or the transfer of fatty acyl ACPs into phospholipid or both. To determine whether apo-ACP affects the synthesis or utilization of long chain acyl ACPs, we uncoupled fatty acid synthesis from phospholipid synthesis. Normally in E. coli, these two processes are tightly coupled and no detectable pool of long chain acyl ACPs exists, thus precluding direct measurement of the rate of fatty acid synthesis. To avoid this problem, we used a system developed recently to uncouple fatty acid and phospholipid biosynthesis (Cho and Cronan, 1995), which utilizes a plasmid encoding a mutant cytosolic E. coli thioesterase I under control of the arabinose promoter. The mutant thioesterase cleaves long
chain acyl-ACPs, resulting in accumulation of large amounts of free fatty acids, and allows direct measurement of the fatty acid biosynthetic rate. The rate of incorporation of labeled acetate into free fatty acids in strains containing the thioesterase plasmid (Fig. 6) did not decrease during low level (−8-fold) ACP overproduction. In fact, the rate of synthesis was increased about 5–6-fold upon ACP overexpression. The free fatty acid pool produced under these conditions consisted primarily of full-length fatty acids, indicating that ACP overproduction failed to block fatty acid biosynthesis. These data indicate that the observed inhibition of lipid synthesis must be the result of inefficient utilization of the full-length fatty acids. Consistent with this hypothesis, induction of cytosolic thioesterase I in a strain that overproduced ACP at a low level (−8-fold) resulted in a complete cessation of growth, whereas neither thioesterase production nor 8-fold ACP overproduction alone were lethal. Thus, it seemed that decreased utilization of acyl-ACP coupled with the hydrolysis of acyl-ACP results in complete growth inhibition.

Apo-ACP Inhibits G3P Acyltransferase In Vitro—G3P acyltransferase catalyzes the transfer of long chain fatty acids from ACP (or CoA) to the 1-position of G3P. Previous work from this laboratory demonstrated in vitro inhibition of G3P acyltransferase activity by holo-ACP (Rock et al., 1981). Since this inhibition did not require a free sulfhydryl group (Rock et al., 1981), it seemed possible that apo-ACP might also inhibit G3P acyltransferase. Therefore, we examined the effect of purified apo-ACP on the activity of G3P acyltransferase in vitro. Apo-ACP was a strong inhibitor of G3P acyltransferase activity (Fig. 7A). However the degree of inhibition varied according to the type of fatty acid donor. Utilization of cis-vaccenoyl-ACP was inhibited to a much greater extent than was utilization of palmitoyl-ACP. Apo-ACP inhibition of the utilization of palmitoleoyl-ACP

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**Fig. 4.** Effects of CoA pool size on ACP overexpression. Panel A, effects of CoA pool size on toxicity of ACP overexpression. Strain DK739 (containing the plasmid with an inducible acpP gene and the feedback-resistant pantothenate kinase) was grown to early log phase. IPTG was then added and growth was followed by measuring turbidity. ■, DK739 plus 1 mM pantothenate without induction; ■, DK739 plus 1 mM pantothenate with induction; ○, DK739 plus 0.1 μM pantothenate without induction; ▲, DK739 plus 0.1 μM pantothenate with induction. Panel B, effects of CoA pool size on ACP species produced during ACP overexpression. Strains DK574 (containing inducible acpP plasmid) and DK739 (which contains the inducible acpP plasmid plus the feedback-resistant mutant pantothenate kinase) were grown to early log phase in the presence of varying amounts of pantothenate to early log phase. IPTG was then added and amounts of pantothenate were grown to early log phase. IPTG was then added and growth was followed by measuring turbidity.

**Fig. 5.** Lipid synthesis during ACP overproduction. The autoradiograph of an argentation thin layer chromatographic separation of the fatty acid methyl esters is shown. Strain DK552 (containing the inducible acpP) was cultured on minimal E medium as described in “Experimental Procedures.” At early log phase IPTG was added and at various time points a 1 ml sample was removed and cultured in the presence of 50 μCi of [14C]acetate for 10 min. Odd-numbered lanes contain DK552 plus 1 mM IPTG labeled at 0, 20, 40, 60, 120, and 240 min after addition of IPTG, respectively. Even-numbered lanes contain strain DK552 labeled in the absence of IPTG at the same time points.

**Fig. 6.** Free fatty acid synthesis in the presence of ACP overproduction. Strains DK757 (containing pHC122 carrying inducible leaderless tesA) and DK758 (containing pHC122), and pMR24 which contains the native acpP gene) were grown to early log phase and labeled as described in “Experimental Procedures.” Thiosterase expression was induced by the addition of arabinose (0.4%), and the cells were cultured for 1 h prior to the addition of 5 μCi of [14C]acetate and growth for 10 min. Free fatty acids were then extracted as in “Experimental Procedures” and analyzed by thin layer chromatography and exposed to a Molecular Dynamics PhosphorImager plate for quantitation. Lane 1, strain DK757 plus 0.4% arabinose; lane 2, strain DK757; lane 3, strain DK758 plus 0.4% arabinose; lane 4, strain DK758. The presence or absence of TesA or ACP overproduction (O.P.) is shown at the bottom of the figure.
The activity of apo-ACP on the second step of phosphatidic acid synthesis, the 1-acyl-G3P acyltransferase reaction, was tested in vitro. Purified apo-ACP was also found to inhibit the ACP-dependent acylation of 1-acyl-G3P acyltransferase (Fig. 7B). However, this inhibition differed considerably from the effect on G3P acyltransferase activity that the inhibition only occurred at much higher apo-ACP concentrations. Moreover, utilization of palmitoyl-ACP was inhibited to a greater extent than was utilization of unsaturated substrates.

Inhibition of Exogenous Fatty Acid Utilization—To determine if the inhibition of the G3P acyltransferases that was observed in vitro also occurred in vivo, incorporation of labeled fatty acid into phospholipids was measured under conditions that did not require de novo synthesis of the fatty acyl donors. Phospholipids were extracted from cells labeled with exogenous palmitate under conditions of ACP overexpression (Fig. 8). Overproduction of ACP resulted in a decrease in utilization of fatty acids. It should be noted that exogenous fatty acids are activated and utilized as CoA esters, suggesting that the inhibition seen in vivo occurs without regard for carrier molecule linked to the fatty acid.

Conclusions—We have shown that the apo form of ACP is a potent inhibitor of lipid metabolism synthesis. It is unclear why apo-ACP is a more powerful inhibitor than holo-ACP. The folding of apo-ACP has been shown to be less stable than that of holo-ACP (J.ackowski and Rock, 1983), but no interaction of the prosthetic group with holo-ACP residues other than the covalent bond to serine 36 has been detected by NMR (Holak et al., 1988). However, we have recently determined that a detectable pool of apo-ACP accumulates in cells with depleted CoA pools as a result of pantothenate starvation under certain growth conditions.3 The specificity of apo-ACP inhibition in regard to acyl donors and the limited number of affected enzymatic activities suggest that apo-ACP might act as a regulator of acyltransferase activity during pantothenate starvation. Because so little is known concerning the enzymes (holo-ACP synthase and ACP phosphodiesterase) that add and remove the prosthetic group, it seems possible that turnover of ACP is regulated by unknown factors and that the interconversion of

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the apo and holo forms of ACP could play an important regulatory role.

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