The Enigmatic Acyl Carrier Protein Phosphodiesterase of Escherichia coli

GENETIC AND ENZYMEOLOGICAL CHARACTERIZATION

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The acyl carrier proteins (ACPs) of fatty acid synthesis are functional only when modified by attachment of the prosthetic group, 4′-phosphopantetheine (4′-PP), which is transferred from CoA to the hydroxyl group of a specific serine residue. Almost 40 years ago Vagelos and Larrabee (Vagelos, P. R., and Larrabee, A. (1967) J. Biol. Chem. 242, 1776–1781) reported an enzyme from Escherichia coli that removed the prosthetic group. We report that this enzyme, called ACP hydrolyase or ACP phosphodiesterase, is encoded by a gene (*yajB*) of previously unknown function that we have renamed *acpH*. A mutant *E. coli* strain having a total deletion of the *acpH* gene has been constructed that grows normally, showing that phosphodiesterase activity is not essential for growth, although it is required for turnover of the ACP prosthetic group in vivo. ACP phosphodiesterase (*Acph*) has been purified to homogeneity for the first time and is a soluble protein that very readily aggregates upon overexpression in vivo or concentration in vitro. The purified enzyme has been shown to cleave acyl-ACP species with acyl chains of 6–16 carbon atoms and is active on some, but not all, non-native ACP species tested. Possible physiological roles for AcpH are discussed.

Acyl carrier proteins (ACPs) are small (<80 residues), highly acidic proteins that are modified by covalent attachment of 4′-phosphopantetheine (4′-PP) that is transferred from CoA and attached via a phosphodiester linkage to the hydroxyl group of a specific serine residue located in the midst of the protein sequence (1). The paradigm ACPs are those of bacterial fatty acid synthesis where the sulphhydril group of the 4′-PP moiety carries the growing fatty acid chain (1). In addition to bacteria, ACPs are found in plant plastids (2), mitochondria (3), and the apicoplasts of apicomplexan parasites (4). The 4′-PP moiety is attached to the apo form of ACP by enzymes called 4′-phosphopantetheinyl transferases (5). *Escherichia coli* AcpS (ACP synthase) is the first such enzyme described (6) and is the only 4′-PP transferase studied physiologically (7, 8).

Proteins similar to the ACPs of fatty acid synthesis are found in polyketide and nonribosomal polypeptide synthesis where these proteins (called PKS ACPs and PCPs, respectively) perform analogous carrier functions (5). Indeed, these ACP-like proteins have the same general four-helix bundle structure predicted (9) and subsequently demonstrated (10–12) for *E. coli* ACP. Soon after the identification of the prosthetic group of ACP as 4′-PP and of 4′-PP transferase activity, Vagelos and Larrabee (13) reported an activity that removed the 4′-PP moiety of ACP in *E. coli*. The enzyme (called ACP hydrolase and now called ACP phosphodiesterase) was partially purified and shown to require a divalent ion and produce apo-ACP and 4′-PP (13). The enzyme was inactive on peptide fragments of ACP suggesting that it recognized the folded structure of ACP (13). Although crude preparations were occasionally used to convert holo-ACP to apo-ACP, this paper remained the only study of this enzyme for 23 years until Fischl and Kennedy (14) reported its purification to apparent homogeneity. These workers showed that ACP phosphodiesterase was an unusually stable protein of M, 25,000 and reported an N-terminal sequence attributed to the enzyme protein. Unfortunately, the N-terminal sequence was found to be that of a flavin-containing protein that lacked phosphodiesterase activity (15) and which has recently been shown to be an azoreductase (15). It seems that Fischl and Kennedy (14) had determined the sequence of a major contaminating protein rather than that of the phosphodiesterase. Regrettably based on this erroneous sequence attribution, many genes (often called *acpD*) have been annotated in bacterial genomes as encoding ACP phosphodiesterase rather than azoreductase.

The discovery of ACP phosphodiesterase activity logically raised the question of whether or not the prosthetic group of ACP turns over independently of the protein moiety. This was soon shown to be the case (16), although interpretation of these data were complicated by the large pools of CoA and its thioesters. Elegant deuterium labeling experiments subsequently showed that the rate of ACP prosthetic group turnover depended on the intracellular concentration of CoA (17). At low CoA levels, prosthetic group turnover was four times faster than the rate of new synthesis of the protein moiety, but at the higher coenzyme A concentrations characteristic of logarithmic growth, turnover was an order of magnitude slower, amounting to ~25% of the ACP pool per generation (17).

We report the isolation of the gene encoding the ACP phosphodiesterase of *E. coli*. We have named the gene *acpH* based on the original enzyme name given by Vagelos and Larrabee (13) to prevent overlap with the mistaken AcpD annotations. The protein has been purified to homogeneity and its substrate specificity studied. The *acpH* gene has also been deleted from the *E. coli* chromosome and the consequences of loss of the enzyme studied.

**EXPERIMENTAL PROCEDURES**

**Materials**—Restriction enzymes and T4 DNA ligase were purchased from New England Biolabs (Beverly, MA). Oligonucleotides for PCR were purchased from IDT (Coralville, IA). The Copy Control™ Fosmid Library Production Kit and MaxPlax Lambda packaging extracts were from Epicenter (Madison, WI). Molecular biology reagents were from...
### TABLE ONE

#### Bacterial strains, plasmids, and oligonucleotides used in this work

| Strain/plasmid | Relevant characteristics | Source |
|---------------|-------------------------|--------|
| **Strain**    |                         |        |
| MG1655        | *Escherichia coli* K12 wild type | Lab collection |
| EPI300        | recA1 endA1 araD139 Δ(ara, leu)/7697 tetA | Epicentre |
| MC1061        | araD139 Δ(ara, leu)-7696 Δ(lac)X74 rpsL hsdR2 | Ref. 49 |
| JT1           | MC1061 acpH-cat | This work |
| SJ16          | pSJ16 acpH-cat | This work |
| JT2           | BL21 DE3 | Invitrogen |
| **Plasmids**  |                         |        |
| pMS421        | pGIB2 carrying the lacI allele | Ref. 50 |
| pMR19         | pKK223-3 carrying *E. coli* acpP under control of the tac promoter | Ref. 51 |
| pCC1FOS       | Cm<sup>+</sup> cosmid with ori and oriV origins of replication | Epicentre |
| pJT1          | pCC1FOS carrying a 40-kb insert containing acpH | This work |
| pJT2          | pCC1FOS carrying a 2.3-kb insert containing acpH subcloned from pJT1 | This work |
| pKD46         | bla pBAD gam bet exo pSC101 oriTS | Ref. 20 |
| pET101D       | Amp<sup>+</sup> Expression vector | Invitrogen |
| pET4          | pET101D encoding AcpH with a C-terminal hexahistidine tag | This work |
| pBAD322       | Amp<sup>+</sup> pBAD expression vector having the complete origin of pBR322 | This work |
| pJT5          | pBAD322 carrying acpH | This work |
| pNRD70        | *A. aeolicus* ACP gene cloned in pBAD322 | Footnote 6 |
| pNRD71        | *B. subtilis* ACP gene cloned in pBAD322 | Footnote 6 |
| pNRD73        | *L. lactis* ACP gene cloned in pBAD322 | Footnote 6 |
| pNRD75        | *B. taurus* mitochondrial ACP gene cloned in pBAD322 | Footnote 6 |
| pNRD136       | *B. subtilis* sfp cloned in pDHK29 | Footnote 6 |
| **Oligonucleotides** | **Sequence (S’–S’<sup>3</sup>)** | |
| CCI1Fos-F     | AATTCGAGCTCGGTACCCC |        |
| CCI1Fos-R     | TGCAAGGTCGACTCTTAGAG |        |
| yajB-pET101-F | CACCATGATTTCATTCGCTCACCCTG |        |
| yajB-pET101-R | TTATGAGTAGATGATGATGATAGTAACCCGTGCCTGAC |        |
| yajB-pBAD-F   | CCAACAGGCTACCATGATTTTATTCGCTACCTG |        |
| yajB-pBAD-R   | GGACACCGTCGATTATACGCCTTGCCTGAC |        |
| yajB-Kcm-F    | CGCTCTTCCCTGCAACCTTTTCCCTACCCCTTTTGATGAATATATAATCTGTTAGCTGAGCTGGAGCTGCTTGA |        |
| yajB-Kcm-R    | TGGCTTCTTCCCTGCAACCTTTTCCCTACCCCTTTTGATGAATATATAATCTGTTAGCTGAGCTGCTTGA |        |
| yajB-chk-F    | GGATGCTCTAGTGCCTGCGG |        |
| yajB-chk-R    | CAACACAGTACGCGCCGTGATC |        |

*J. Cronan, unpublished results.*

Qiagen (Valencia, CA). The pET101D TOPO Expression cloning kit was from Invitrogen. VivaSpin D columns were obtained from Viva-science (Edgewood, NY). β-[3–H]Alanine and [35S]methionine were purchased from American Radiolabeled Chemicals (St. Louis, MO). Other chemicals, unless otherwise stated were from Sigma.

**Bacterial Strains and Plasmids**—All strains used were derivatives of *E. coli* K12. Strains MG1655, BL21, and MC1061 derivatives were grown on LB at 37 °C (TABLE ONE). Strain SJ16 and its derivatives were grown on minimal E medium (18) with 0.4% glucose or glycerol, as carbon source, 0.001% thiamine, and 0.1% casamino acids (Difco). When added, ampicillin was used at 100 μg/ml, spectinomycin at 50 μg/ml, and kanamycin at 30 μg/ml.

**Construction and Screening of the Cosmid Library**—An expression library was constructed essentially according to the method described previously (19). Strain MG1655 DNA purified using the Wizard genomic DNA purification kit (Promega) was sheared to ~30–50-kb fragments by repeated pipetting. Sheared DNA was then end repaired to generate blunt ends using T4 DNA polymerase and run on a 1% low melting point agarose gel. Fragments ~40 kb in size were excised, DNA was recovered from the gel and ligated to Eco72I-digested pCC1FOS. The ligation mixture was size-selected by packaging into λ phage particles and used to infect EPI300 to chloramphenicol resistance. Transformants were restreaked and separated into sets of 50 based on colony size. Individual cultures of each set of colonies were grown overnight in LB/chloramphenicol, pooled together, and diluted to *A*<sub>600</sub> of 0.2 in 100 ml of the same medium. Each pooled culture was grown to *A*<sub>600</sub> of 0.6 and induced with 0.02% arabinose for a further 4 h. The cells were washed with an equal volume of AcpH storage buffer (50 mM Tris-HCl, pH 7.5, 10 mM MgCl<sub>2</sub>, 2 mM MnCl<sub>2</sub>, 5 mM DTT, 10% glycerol, and 0.2 mM phenylmethylsulfonyl fluoride), resuspended in 1 ml of the same buffer, and lysed by sonication. Cell-free extracts were assayed for AcpH activity as described above. The pool whose extract showed a level of AcpH activity above that of the vector control was further subdivided into 10 sets of five colonies each and pooled cultures were grown, induced, and lysed as before. In the final screen of the library extracts were made and assayed from cultures grown from single colonies. Cosmid DNA was isolated from an induced culture of the clone (pJT1) expressing AcpH (Qiagen Large Construct kit) and used to transform strain EPI300. Upon confirmation of increased activity, the encoding gene was subcloned from cosmid pJT1 as follows. The cosmid DNA was...
diluted to 20 μg/ml in 10 mM Tris-HCl containing 1 mM EDTA (pH 8.0) buffer and sonicated for 4 s at the lowest power setting. The sheared DNA was end-repaired as above and fractionated on a 1% agarose gel. Fragments of 2–3 kb were extracted from the gel and ligated to Eco721-digested pCC1fos. The ligation mixture was used to transform EPI300, and 200 clones were screened as before. Cosmid DNA was purified from clones that showed increased AcpH activity and sequenced with primers CC1Fos-F and CC1Fos-R.

**DNA Manipulations**—The sequences of the primers used are given in TABLE ONE. The acpH gene was PCR amplified from pJT1 using primers yajB-pET101-F and yajB-pET101-R. The resulting PCR product was cloned into the TOPO vector pET101D to give plasmid pJT4 in which a C-terminal His-tagged AcpH was expressed from a T7 promoter. The acpH sequence was also PCR-amplified using primers yajB-pBAD-F and yajB-pBAD-R and the KpnI- and PstI-digested PCR products were cloned into pBAD322 digested with the same enzymes to give plasmid pJT5. Strain JT1 was constructed using the λ Red-mediated recombination system (20). The chloramphenicol acetyltransferase (cat) gene from plasmid pKD3 was amplified using primers yajB-KCm-F and yajB-KCm-R and electroporated into strain MC1061 containing the helper plasmid pKD46 resulting in replacement of the entire coding sequence of acpH with the cat gene. Chloramphenicol-resistant colonies were verified by colony-PCR using primers yajB-chk-F and yajB-chk-R. Strain JT2 was constructed by using a P1 lysate made on JT1 to transduce SJ16 to chloramphenicol resistance. The acpH* genotype was verified by assaying crude extracts of both strains for AcpH activity as described below.

**Purification of ACP Species**—Holo-ACP was purified using a modified procedure based on the methods described previously (21, 22). Strain DK574 (which is SJ16 carrying plasmids pMS421 and pMR19) was grown to an A600 of 0.8 and induced with 1 mM isopropyl-D-thiogalactopyranoside for a further 30 min and dried at 40 °C. The dried gels were exposed to pre-irradiated cassette and placed in a cassette for autoradiography.

**ACP Phosphodiesterase**—Strain SJ16 derivatives were grown in minimal medium containing 0.5 mM β-alanine overnight to reduce the coenzyme A pools. Overnight cultures were subcultured in minimal medium with 0.5 mM [3H]β-alanine (1 Ci/mmol) until they reached an A600 of 1.0. Cells were pelleted and washed twice in minimal medium and then diluted 5-fold in the same medium containing 8 μM unlabeled β-alanine. 0.5-ml samples were withdrawn at various time points, and cell extracts were analyzed on a 12% SDS-polyacrylamide gel. The cell suspension was lysed by shaking at room temperature for 1 h and then centrifuged. AcpH was purified from the supernatant on Ni-NTA-agarose under denaturing conditions as follows. The supernatant was applied to a column of Ni-NTA-agarose and the column was washed and eluted with buffers of the same composition but decreasing pH: wash buffer 1, pH 6.3; wash buffer 2, pH 5.9; and elution buffer, pH 4.5. Fractions were assayed for AcpH activity.

**Expression and Purification of C-terminal Hexahistidine-tagged AcpH**—Strain BL21 DE3 (pJT4) was grown in LB-ampicillin to A600 of 0.8 and induced with 1 mM isopropyl-D-thiogalactopyranoside for a further 4 h. The cells were washed in an equal volume of 50 mM sodium MES, pH 6.1, and concentrated 20-fold in the same buffer. Crude extracts were prepared by sonication and an equal volume of ice-cold isopropl alcohol was added to the extract and incubated with stirring at 4 °C for 1 h. The suspension was centrifuged, and the supernatant was applied to a column of Ni-NTA-agarose and the column was washed and eluted with buffers of the same composition but decreasing pH: wash buffer 1, pH 6.3; wash buffer 2, pH 5.9; and elution buffer, pH 4.5. Fractions were assayed for AcpH activity.

**Acyl-ACP Synthesis**—Acyl-ACPs were synthesized according to the method of Shen et al. (26). Reactions contained 100 mM Tris-HCl, pH 7.8, 10 mM MgCl2, 10 mM ATP, 1 mM DTT, 50 μM holo-ACP, 80 μM concentrations of each fatty acid, and 50 nM purified *Vibrio harveyi* acyl-ACP synthetase in a 50-μl volume and were incubated at 37 °C for 4 h. Essentially complete conversion to the acylated forms was shown by gel electrophoresis.

**In Vitro Assay of AcpH Activity**—AcpH activity was assayed essentially by the method of Fischl and Kennedy (14). The assay was performed at 25 °C and contained 50 mM Tris-HCl, pH 8.6, 0.02 mM MnCl2, 25 mM MgCl2, 1 mM DTT, and 10–50 μM acyl carrier protein substrate in a final volume of 50 μl. Reactions were initiated by the addition of 20 μg of crude extract protein or 1–200 nM purified AcpH and terminated after 60 min by the addition of trichloroacetic acid to 6%. The precipitate was washed in 6% trichloroacetic acid and resuspended in 50 μl of 0.5 M Tris-HCl, pH 6.8. 10 μl of this was analyzed by conformationally sensitive gel electrophoresis with detection by staining with Coomassie Brilliant Blue R-250. When analyzed by mass spectrometry, the reaction mixtures were first dialyzed against 2 mM ammonium acetate with two buffer changes and then dried under vacuum. Radioactive AcpH assays were performed under the same conditions except that 15–40 μl [3H]holo-ACP or 3–15 μg of protein from an extract of β-alanine-labeled cells was used as substrate. The reactions were either analyzed by fluorography as described below or by release of the [3H] label from the protein quantitated as follows: 30 μl of the trichloroacetic acid supernatant was treated with 200 μl of water-saturated diethyl ether to remove the acid and 20 μl of the aqueous phase was then mixed with 5 ml of scintillation fluid and counted in a Beckman LS6500 scintillation counter. For fluorography following electrophoresis, the acylamide gels were fixed in 50% methanol and 10% acetic acid for 30 min then incubated in Amplycon solution (Amersham Biosciences) for a further 30 min and dried at 40 °C. The dried gels were exposed to pre-flashed X-AR Bio-Max films (Kodak) at −80 °C.

**Holo-ACP Turnover**—Strain SJ16 derivatives were grown in minimal medium containing 0.5 mM β-alanine overnight to reduce the coenzyme A pools. Overnight cultures were subcultured in minimal medium with 0.5 mM [3H]β-alanine (1 Ci/mmol) until they reached an A600 of 1.0. Cells were pelleted and washed twice in minimal medium and then diluted 5-fold in the same medium containing 8 μM unlabeled β-alanine. 0.5-ml samples were withdrawn at various time points, and cell extracts were made in 25 mM sodium MES, pH 6.1. Ten μl of each extract was run on 20% polyacrylamide gels and analyzed by fluorography as given above.

**Expression and Purification of C-terminal Hexahistidine-tagged AcpH**—Strain BL21 DE3/pJT4 was grown in LB-ampicillin to A600 of 0.8 and induced with 1 mM isopropyl-D-thiogalactopyranoside for a further 4 h. The cells were washed in an equal volume of lysis buffer (100 mM NaH2PO4, 10 mM Tris-HCl, pH 8.0) and resuspended in one-tenth volume of the same buffer containing 8 μl urea. The cell suspension was lysed by shaking at room temperature for 1 h and then centrifuged. AcpH was purified from the supernatant on Ni-NTA-agarose under denaturing conditions as follows. The supernatant was applied to a column of Ni-NTA-agarose resin, and the column was washed and eluted with buffers of the same composition but decreasing pH: wash buffer 1, pH 6.3; wash buffer 2, pH 5.9; and elution buffer, pH 4.5. Fractions were analyzed on a 12% SDS-polyacrylamide gel, purified AcpH was diluted to a concentration of 10 μg/ml and dialyzed against AcpH storage buffer mentioned above with the addition of 8 μM urea and 50 mM each of L-arginine and L-glutamate to prevent aggregation (27). The molar con-
centration of urea in the arginine-glutamate buffer was decreased stepwise in successive dialysis steps (8, 4, 2, 1, 0.5, and 0) for 2 h each followed by overnight dialysis (with three changes of buffer) against the final buffer.

**Gel Filtration Chromatography**—A 5-ml culture of BL21 DE3 transformed with plasmid pT74 was grown to mid-log phase, treated with 200 μg/ml rifampicin and 42.5 nM l-[³⁵S]methionine (250 μCi) (28). The culture was grown for a further 1 h, concentrated 10-fold, and crude extracts were made in column running buffer (50 mM Tris-HCl, pH 7.5, 10 mM MgCl₂, 0.15 mM NaCl, 5 mM DTT, 50 mM l-glutamate, and 50 mM l-arginine). The extract was dialyzed against the same buffer with two changes to remove soluble label and 0.1 ml of the dialyzed extract was applied to a Superdex 200 column (1 × 44 cm). 0.5-ml fractions were collected at a flow rate of 65 μl/min and 0.1 ml of each fraction was mixed with 5 ml of scintillation fluid and analyzed for radioactivity in a Beckman LS6500 scintillation counter. The standard proteins were run in the same manner except that 20 μg of each purified protein was applied to the column and the fractions were analyzed by the Bradford assay (29).

**RESULTS**

**Expression Cloning of ACP Phosphodiesterase**—Because prior attempts at identification of the gene encoding ACP phosphodiesterase by N-terminal sequencing of the enzyme purified >3000-fold from *E. coli* cell extracts (14) gave the sequence of an unrelated contaminating protein (AzoR) (15), it seemed that the phosphodiesterase is a very non-abundant protein. We therefore took a different approach, that of screening a clone bank for plasmid clones that gave increased phosphodiesterase activity *in vitro*. We had previously used this approach to identify the gene encoding *Enterococcus faecalis* lipoamidase (19). As in that study toxicity of the expressed gene seemed likely to be a problem, as high levels of phosphodiesterase activity would be expected to inhibit growth by depletion of holo-ACP pools required for fatty acid synthesis and by increasing the relative amount of apo-ACP, a potent inhibitor of cell growth (21). Therefore, we used the same vector system (30) as in the lipoamidase work that has the following features. The vector is a cosmid that carries an F-factor origin of replication (*oriV*) and partitioning function that maintains the cosmid clones in single copy under uninduced conditions. This alleviates the problem of under-representation of clones carrying genes that are toxic in multiple copies. A second origin of replication, *oriV*, is also present on the cosmid that requires the TrfA protein for function. The host strain carries a copy-up allele of the *trfA* gene (30) integrated into the chromosome under control of a pBAD promoter. Induction with arabinose causes expression of TrfA allowing initiation of replication from the *oriV* origin to give a 40–80-fold increase in the cosmid copy number. This inductive increase in copy number is useful both to increase yields during isolation of cosmid DNA and more importantly, to increase expression of genes of cosmid DNA and more importantly, to increase expression of genes. An increase in copy number is useful both to increase yields during isolation of cosmid DNA and more importantly, to increase expression of genes.

**Cosmid DNA was isolated from clone SA1, transformed into EPI300, and gave increased phosphodiesterase activity. To locate the gene further, the cosmid (designated pT72) was ligated into the vector pCC1FOS (2).** Two hundred subclones were streaked from the resulting transformants and divided into sets of 10, the pooled cultures were induced and crude extracts were screened as before. After two rounds of screening, a single clone JT1525 was identified as carrying a small cosmid (designated pT72) that gave ACP phosphodiesterase expression (Fig. 1C).
spanned a region from nucleotide 422807 (within the *malZ* gene) to 425159 (within the *queA* gene). The insert contained 749 bp of *malZ*, 924 bp of *queA*, and the complete *yajB* open reading frame (582 bp) that is currently annotated in GenBank™ as encoding a putative glycoprotein of uncharacterized function. The calculated molecular weight of 22,961 for the encoded protein agreed well with the findings of Fischl and Kennedy (14) who recovered phosphodiesterase activity from the 25,000 region of SDS gels (14). The original name given to ACP phosphodiesterase was ACP hydrolyase (13) and thus we have renamed *yajB* as *acpH* to acknowledge this precedent. Protein-protein BLAST searches in GenBank revealed that AcpH has homologues only in Gram-negative bacteria and mainly among the *H.9253*-proteobacteria (Fig. 2), none of which has a biochemically characterized function.

Strain JT1, in which the entire coding sequence for *acpH* was replaced by the *cat* gene, was constructed by homologous recombination as described under “Experimental Procedures.” This strain demonstrated no noticeable growth phenotype when grown in rich or minimal media. Crude extracts of JT1 were prepared from log phase cultures and assayed for phosphodiesterase activity that was undetectable both by the gel shift (Fig. 3) and [3H]4,11032-PP release (≤1% of the activity of the wild type extract) assays. Strain MC1061 was also transformed with plasmid pJT5 carrying *acpH* under pBAD control. Crude extracts made from an arabinose-induced culture of this strain were assayed for AcpH activity (Fig. 3). As expected, this strain exhibited about 2-fold greater levels of AcpH activity than the vector control strain (higher levels of expression were precluded by aggregation of the protein).

**Expression and Purification of AcpH**—The *acpH* gene was PCR amplified from pJT2 and cloned into the TOPO expression vector pET101D (Invitrogen) to encode a protein that had a C-terminal hexahistidine tag and was expressed under control of a T7 promoter. This plasmid (called pJT4) was then transformed into strain BL21(DE3). Although crude extracts of the transformants showed overexpressed AcpH activity both under induced and non-induced conditions (data not shown), the bulk of the protein was insoluble. Fischl and Kennedy (14) had a similar aggregation problem during their protein purification. Altering isopropyl β-D-thiogalactopyranoside concentrations, growth temperatures, or use of N-terminal fusion tags (thioredoxin and maltose binding protein) previously reported to increase solubility of expressed proteins (32, 33) failed to alleviate the aggregation problem.

The hexahistidine-tagged AcpH was readily purified from inclusion bodies under denaturing conditions (Fig. 4) but the protein aggregated during refolding from urea. However, when 50 mM 1-arginine and 50
Fractions E6, E7, and E8 were pooled, diluted to 10 μg/ml, and refolded as described under “Experimental Procedures.”

Because the purified protein remained soluble only at low concentrations, we examined dialyzed crude extracts in which Acpl expressed from a T7’ promoter was specifically labeled with [35S]L-methionine in the presence of rifampicin (28, 34). The major labeled peaks in the included volume of the column eluted in essentially the same fractions as did chymotrypsinogen and bovine serum albumin (Fig. 5), suggesting that monomeric and trimeric species are present. A major fraction of the 35S label eluted in the void volume of the column, presumably because of aggregation of Acpl during the dialysis needed to remove unincorporated [35S]methionine. We attempted to decrease aggregation by lowering expression levels, but the void volume material appeared even when only the basal level (uninduced) of expression was used for specific labeling of Acpl (Fig. 5). Moreover, use of the glutamate/arginine mixture during both dialysis and chromatography failed to prevent the appearance of labeled protein in the void volume.

The Protein Product of Acpl Is Apo-ACP—To verify that Acpl encodes a phosphodiesterase and not, for example, a protease giving products that have an altered mobility on non-denaturing gels, purified holo-ACP (Fig. 6A) was treated with purified Acpl and the products were analyzed by electrospray ionization mass spectrometry (Fig. 6B). The observed mass of the reaction product, 8508.4 Da, was in excellent agreement with the calculated mass of apo-ACP (8508.33 Da). The reaction product was also a substrate for phosphopantetheinyl transfer from CoA catalyzed by AcpS (Fig. 6C), thereby confirming the product to be apo-ACP. Prior workers have demonstrated the other product to be 4′-PP (13).

Acpl Is Responsible for ACP Prosthetic Group Turnover in Vivo—We then sought to clarify the role of Acpl in ACP prosthetic group turnover in vivo by performing turnover experiments with derivatives of strain S16, which carries a panD2 deletion and requires externally supplied β-alanine for growth on minimal medium (35), allowing radiolabeling of the 4′-phosphopantetheinyl group of CoA and holo-ACP pools by growth on [3H]β-alanine. Strains S16 and JT2 (S16 acpH::cat) were depleted of CoA by starvation for β-alanine (see “Experimental Procedures”) then subcultured in minimal medium containing 0.5 μM [3H]β-alanine (1 Ci/mol) to early stationary phase. The cells were then collected, washed, and shifted to medium containing unlabeled β-alanine (8 μM). Samples (0.5 ml) of each culture were withdrawn at regular time intervals. Crude extracts were then made and analyzed by gel electrophoresis followed by fluorography. In agreement with a previous study (17), turnover in strain SJ16 was most rapid within the first 10 min of recovery from β-alanine starvation (Fig. 7A). After this time however, the amount of label failed to decrease, probably because the CoA pool had not been sufficiently depleted for efficient chase with unlabeled β-alanine. In contrast, the amount of 3H label bound to ACP in strain JT2 remained relatively constant (Fig. 7B) over the time course of the experiment. These results indicate that deletion of acpH abolishes ACP prosthetic group turnover in vivo. Jackowski and Rock (35, 36) have reported that the 4′-PP from ACP prosthetic group turnover is excreted to the culture medium and cannot be recovered by the cells. We therefore followed the excretion of β-[3H]alanine-labeled metabolites during the chase period (Fig. 7C) and found, as expected, that labeled metabolite excretion was significantly decreased in the mutant strain. Vallari and Jackowski (37) have reported that 4′-PP can be directly derived from degradation of CoA and have argued that this pathway does not involve ACP phosphodiesterase. We have assayed the labeled material excreted by strain JT2 by thin layer chromatography and our preliminary results indicate that a major fraction of
the excreted material has the chromatographic properties of 4′-PP consistent with the report of Vallari and Jackowski (37).

Prosthetic group turnover was also examined in a derivative of strain SJ16 that overexpressed AcpH from plasmid pJT5. However, in this case, the turnover profile resembled that of the vector control (data not shown). This is presumably because an increase in prosthetic group turnover rate does not increase excretion of the label and reattachment of 4′-PP by AcpS maintains the level of labeled ACP. However, it should be noted that we could only very modestly overproduce AcpH because higher level production resulted in intracellular aggregation of the protein.

**Substrate Specificity of AcpH**—Vagelos and Larrabee (13) reported that partially purified AcpH cleaves acetyl-ACP at essentially the same rate as ACP. To determine whether or not AcpH was also capable of hydrolyzing ACP species having acyl groups of greater chain length, acyl-ACP substrates having fatty acyl chain lengths from C-6 to C-16 were synthesized from the free fatty acids, ACP, and purified V. harveyi acyl-ACP synthetase (26). The acyl-ACP substrates were treated with AcpH as described, and reaction products were analyzed on 20% polyacrylamide gels containing 2.5 M urea (Fig. 8). AcpH hydrolyzed all of the acyl-ACP thioesters tested. Note that no holo-ACP was formed in these reactions, indicating that the acyl thioester linkages stayed intact during the assay. Although the results are not quantitative, the rate of hydrolysis appears to decrease with increasing chain lengths. The enzyme therefore appears to demonstrate a preference for the unacylated ACP and short chain fatty acyl-ACPs over the medium and long chain species.

The ability of AcpH to cleave 4′-PP from the ACPs of other species was also examined. As described under “Experimental Procedures,” strain SJ16 transformed with plasmids carrying genes encoding putative fatty acid synthesis acyl carrier proteins from *Aquifex aeolicus*, *Bacillus subtilis*, *Lactococcus lactis*, and the mitochondrial ACP of *Bos taurus* under control of the *E. coli* PBAD promoter were depleted of CoA, then grown in medium containing 0.5 mM [3H]alanine (1 Ci/mol) to early stationary phase. The cells were then collected by centrifugation, washed, and shifted to medium containing 8 μM unlabeled β-alanine. Samples (0.5 ml) of the cultures were taken at regular intervals, crude extracts were made, and a fixed volume of each sample was analyzed by gel electrophoresis and fluorography (see “Experimental Procedures”). C, at each time point, 10-μl aliquots of the culture media of strains SJ16 (A) and JT2 (A) were mixed with 5 ml of scintillation fluid and counted for excreted [3H]label.
hydrolyzed the 4′-PP prosthetic group from the ACPs of *A. aeolicus* and *B. subtilis* but not those of *L. lactis* or *B. taurus* (note that all lanes contain the host ACP band). Sequence alignments of the regions flanking the phosphopantetheinylated serine are shown in Fig. 9B. These regions, in particular helix 2 of ACP, have been implicated as the primary determinants for substrate specificity of AcpS for carrier proteins (39, 43, 44). It is interesting to note that although the ACPs of *B. subtilis* and *A. aeolicus* are structurally similar to *E. coli* ACP and are substrates of *E. coli* AcpH, our searches failed to detect *acpH* homologs in these species.

**DISCUSSION**

Although ACP phosphodiesterase was discovered almost 40 years ago (13), the enzyme had not been purified to homogeneity and there was no valid information on the gene(s) that encode the enzymatic activity. We have accomplished both of these aims. The encoding gene was no valid information on the gene(s) that encode the enzymatic activity. We have accomplished both of these aims. The encoding gene was no valid information on the gene(s) that encode the enzymatic activity. We have accomplished both of these aims. The encoding gene was no valid information on the gene(s) that encode the enzymatic activity. We have accomplished both of these aims. The encoding gene was no valid information on the gene(s) that encode the enzymatic activity. We have accomplished both of these aims. The encoding gene was no valid information on the gene(s) that encode the enzymatic activity. We have accomplished both of these aims. The encoding gene was no valid information on the gene(s) that encode the enzymatic activity. We have accomplished both of these aims. The encoding gene was no valid information on the gene(s) that encode the enzymatic activity. We have accomplished both of these aims. The encoding gene was no valid information on the gene(s) that encode the enzymatic activity. We have accomplished both of these aims. The encoding gene was no valid information on the gene(s) that encode the enzymatic activity. We have accomplished both of these aims. The encoding gene was no valid information on the gene(s) that encode the enzymatic activity. We have accomplished both of these aims. The encoding gene was no valid information on the gene(s) that encode the enzymatic activity. We have accomplished both of these aims. The encoding gene was no valid information on the gene(s) that encode the enzymatic activity. We have accomplished both of these aims. The encoding gene was no valid information on the gene(s) that encode the enzymatic activity. We have accomplished both of these aims. The encoding gene was no valid information on the gene(s) that encode the enzymatic activity. We have accomplished both of these aims. The encoding gene was no valid information on the gene(s) that encode the enzymatic activity. We have accomplished both of these aims. The encoding gene was no valid information on the gene(s) that encode the enzymatic activity. We have accomplished both of these aims. The encoding gene was no valid information on the gene(s) that encode the enzymatic activity. We have accomplished both of these aims. The encoding gene was no valid information on the gene(s) that encode the enzymatic activity. We have accomplished both of these aims. The encoding gene was no valid information on the gene(s) that encode the enzymatic activity. We have accomplished both of these aims. The encoding gene was no valid information on the gene(s) that encode the enzymatic activity. We have accomplished both of these aims. The encoding gene was no valid information on the gene(s) that encode the enzymatic activity. We have accomplished both of these aims. The encoding gene was no valid information on the gene(s) that encode the enzymatic activity. We have accomplished both of these aims. The encoding gene was no valid information on the gene(s) that encode the enzymatic activity. We have accomplished both of these aims. The encoding gene was no valid information on the gene(s) that encode the enzymatic activity. We have accomplished both of these aims. The encoding gene was no valid information on the gene(s) that encode the enzymatic activity. We have accomplished both of these aims. The encoding gene was no valid information on the gene(s) that encode the enzymatic activity. We have accomplished both of these aims. The encoding gene was no valid information on the gene(s) that encode the enzymatic activity. We have accomplished both of these aims. The encoding gene was no valid information on the gene(s) that encode the enzymatic activity. We have accomplished both of these aims. The encoding gene was no valid information on the gene(s) that encode the enzymatic activity. We have accomplished both of these aims. The encoding gene was no valid information on the gene(s) that encode the enzymatic activity. We have accompl

We anticipated that multiple copies of *acpH* would be toxic to *E. coli* because apo-ACP would accumulate to high levels and block cell growth. However, this prediction was incorrect. Strains overproducing AcpH grow only slightly more slowly than wild type strains and this modest effect on growth could be as readily because of the accumulation of protein aggregates as to a direct effect of phosphodiesterase activity.

Indeed, we found no detectable increase in the rate of ACP prosthetic group turnover upon AcpH overexpression (data not shown), which, however, is limited to modest levels of overexpression because of the aggregation of the protein upon overproduction. However, even modest overproduction might be inhibitory because AcpS, the enzyme responsible for attaching the 4′-PP prosthetic group to apo-ACP, is strongly inhibited by apo-ACP in vivo (8). Therefore, a plausible scenario would be that increased levels of apo-ACP engendered by AcpH overproduction would inhibit AcpS and thereby prevent reattachment of the prosthetic group. However, AcpH activity in crude extracts has been reported to be inhibited by physiological concentrations of CoA and acetyl-CoA both of which are substrates of AcpS (40). *E. coli* has two known 4′-PP transferases active on ACP, AcpS and AcpT (6, 8). AcpS is thought to be the enzyme of physiological importance because it is an essential gene (41) and mutants deficient in AcpS activity accumulate apo-ACP (8, 42). If in vivo *E. coli* AcpS is strongly inhibited by an AcpH substrate such as holo-ACP or an acyl-ACP, then the lack of toxicity of AcpH upon overexpression can be rationalized. In this scenario cleavage of the inhibitor would result in a large increase in AcpS activity that would counteract AcpH action and thereby give the constant holo-ACP pool we observe. Therefore, AcpH and AcpS may be prevented from forming a complete futile cycle by inhibition of one enzyme by the substrates of the other. We are currently investigating this possibility. It seems likely that AcpH activity is somehow modulated in vivo because based on the specific activity of purified AcpH and the cellular level of the protein measured by use of a strain carrying an epitope-tagged chromosomal *acpH* gene that the cellular content of AcpH activity is suffi-

**FIGURE 9.** Activity of *E. coli* AcpH toward ACPs of other species. Panel A, derivatives of strain SJ16 transformed with plasmids carrying the genes encoding the *A. aeolicus*, *B. subtilis*, *L. lactis*, and *B. taurus* mitochondrial ACP on pBAD222 were starved for CoA overnight and subcultured in minimal medium with 1.0 μM [3H]3′-PP-asparagine (1 Ci/mol) and 0.2% arabinose. The strain carrying *L. lactis* ACP also carried plasmid pDHK29 encoding Sfp, which was induced with 10 μM isopropyl β-D-thiogalactopyranoside. Crude extracts were made of which one-half was treated with AcpH and the other left untreated, and AcpH activity was followed by loss of the labeled bands. The reaction products were analyzed by gel electrophoresis followed by fluorography (see “Experimental Procedures”). Each well received 3 μg of total protein. Strain SJ16 transformed with pBAD222 was used as a vector control and as a reference for *E. coli* ACP expressed from the chromosome in all extracts (lanes marked Control). Panel B, sequence alignments of conserved regions surrounding the phosphopantetheinylated serine of the acyl carrier proteins in panel A. The alignments were generated using Clustal W. Sequence identities are shaded in black and similarities in gray. The asterisk denotes the phosphopantetheinylated serine and the bracket denotes the helix 2 region.

**FIGURE 8.** AcpH utilizes acyl-ACP substrates. The acyl-ACPs were synthesized using free fatty acids and *V. harveyi* acyl-ACP synthetase as described under “Experimental Procedures.” One-half of the acyl-ACP was treated with AcpH and the other half was left untreated. The AcpH reaction mixtures contained 5 μM acyl-ACP and 33 nM AcpH. Holo-ACP was also treated with AcpH as a positive control.
cient to hydrolyze all of the cellular ACP in <1 min. Hence there is clearly sufficient AcpH activity to account for the rate of prosthetic group turnover seen in vivo.

It seems interesting that thus far only those ACPs that are good substrates for AcpS in their apo forms are substrates for AcpH in their modified forms, suggesting that the two enzymes recognize the same or very similar ACP features. From the crystal structure of the B. subtilis AcpS-ACP cocrysal (12) and helix modification experiments (39), it is clear that the important AcpS recognition sites reside in helix 2 of ACP and hence it seems likely that AcpH also recognizes helix 2. Indeed all of the ACPs we tested that were not AcpH substrates have helix 2 sequences that differ markedly from that of E. coli (Fig. 9B).

Despite the progress we report, the physiological role of AcpH remains enigmatic. AcpH seems limited to Gram-negative organisms and we have shown that the enzyme is not essential for growth of E. coli. It also seems that AcpH is not required for growth in the natural environment because two organisms very closely related to E. coli, Shigella dysenteriae, and two strains of Shigella flexneri have an 8-bp deletion (probably because of recombination between two copies of a short directly repeated sequence) within the 5′-end of the gene expected to result in a truncated protein of 50 residues (Fig. 2). Surprisingly, although it should not be expressed because of the shift in reading frame caused by the deletion, translation of the DNA sequence downstream of the deletion gives a protein that is 96% identical to E. coli AcpH (Fig. 2). The same is true of the N-terminal fragment (Fig. 2). The fact that these sequences have not appreciably drifted away from those of E. coli argues that the deletion event was a recent occurrence or that the protein fragments perform some function that provides a selection for their existence.

Given that AcpH is not an essential protein, what is its physiological role? The fact that the protein is found only in Gram-negative organisms and is limited to two organisms very closely related to E. coli K12, the organism we have studied (45, 46). It is interesting that when compared with E. coli the Shigellae seem prone to lose catabolic genes (46) because AcpH can be considered to be an enzyme involved in the catabolism of CoA (by the combined action of AcpS and AcpH CoA is converted to 4′-PP and 3′, 5′-ADP).

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