Holo-(Acyl Carrier Protein) Synthase and Phosphopantetheinyl Transfer in Escherichia coli*

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Holo-(acyl carrier protein) synthase (AcpS) post-translationally modifies apoacyl carrier protein (apoACP) via transfer of 4'-phosphopantetheine from coenzyme A (CoA) to the conserved serine 36 γ-OH of apoACP. The resulting holo-acyl carrier protein (holoACP) is then active as the central coenzyme of fatty acid biosynthesis. The acpS gene has previously been identified and shown to be essential for Escherichia coli growth. Earlier mutagenic studies isolated the E. coli MP4 strain, whose elevated growth requirement for CoA was ascribed to a deficiency in holoACP synthesis. Se- quencing of the acps gene from the E. coli MP4 strain (denoted acpS1) showed that the AcpS1 protein contains a G4D mutation. AcpS1 exhibited a 5-fold reduction in its catalytic efficiency when compared with wild type AcpS, accounting for the E. coli MP4 strain phenotype. It is shown that a conditional acps mutant accumulates apoACP in vivo under nonpermissive conditions in a manner similar to the E. coli MP4 strain. In addition, it is demonstrated that the gene product, YhhU, of a previously identified E. coli open reading frame can completely suppress the acps conditional, lethal phenotype upon overexpression of the protein, suggesting that YhhU may be involved in an alternative pathway for phosphopantetheinyl transfer and holoACP synthesis in E. coli.

Escherichia coli utilizes a repeated cycle of condensation, reduction, dehydration, and isomerization reactions to produce saturated and unsaturated fatty acids (1, 2). This biosynthetic mechanism employs multiple enzymatic activities conglom- erately termed the fatty acid synthase. The central coenzyme in all fatty acid synthases is the holo form of acyl carrier protein. The central coenzyme in mechanism employs multiple enzymatic activities conglomer- ously termed the fatty acid synthase. The central coenzyme in mechanism employs multiple enzymatic activities conglomer- ously termed the fatty acid synthase. The central coenzyme in mechanism employs multiple enzymatic activities conglomer- ously termed the fatty acid synthase. The central coenzyme in mechanism employs multiple enzymatic activities conglomer- ously termed the fatty acid synthase. The central coenzyme in mechanism employs multiple enzymatic activities conglomer- ously termed the fatty acid synthase. The central coenzyme in mechanism employs multiple enzymatic activities conglomer- ously termed the fatty acid synthase. The central coenzyme in mechanism employs multiple enzymatic activities conglomer- ously termed the fatty acid synthase. The central coenzyme in mechanism employs multiple enzymatic activities conglomer- ously termed the fatty acid synthase. The central coenzyme in mechanism employs multiple enzymatic activities conglomer- ously termed the fatty acid synthase. The central coenzyme in mechanism employs multiple enzymatic activities conglomer- ously termed the fatty acid synthase.

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‡ The abbreviations used are: ACP, acyl carrier protein; AcpS, holo- (acyl carrier protein) synthase; PPTase, phosphopantetheinyltrans- ferase; CoA, coenzyme A; 4'-PP, 4'-phosphopantetheine; PAGE, polyacrylamide gel electrophoresis; HPLC, high performance liquid chromatography; PCR, polymerase chain reaction.
### EXPERIMENTAL PROCEDURES

#### Bacterial Strains and Plasmids—

| Strain or plasmid | Relevant characteristics* | Source or Ref. |
|-------------------|---------------------------|----------------|
| W3110             | E. coli K-12 prototroph    | Laboratory collection |
| HT210             | W3110 recO::kan            | 10 |
| HT253             | W3110 pdxS::Δ16Δ17Tn10     | 10 |
| pBR322            |                           | Laboratory collection |
| YH130             | W3110 pYON110              | This work |
| YH137             | HT253 pBAD22               | This work |
| YH138             | HT253 pYON113              | This work |
| YH139             | HT253 pCY314               | This work |
| YH140             | HT253 pYON110              | This work |
| YH141             | HT253 pYON108              | This work |
| RL101             | BL21(DE3) pRL101           | 12 |
| RL102             | BL21(DE3) pRL102           | This work |
| RF101             | MP3 pBR322                 | This work |
| RF102             | MP4 pBR322                 | This work |
| RF103             | MP4 pDLC140                | This work |
| RF104             | MP4 acpS+ (created by MP4 × p1_cry(HT210)) | This work |
| DK554             | MP4 pMMR19 pMS421          | 20 |
| DK675             | SJ16 pDK675                | 20 |
| DK700             | SJ16 pDK685                | 20 |
| TX2004            | JC7623 acpS::MudJ2         | 11 |
| pBAD22            | Ap′ araBAD promoter expression vector | 19 |
| pBR322            | Ap′ Te′                  | Laboratory collection |
| pACYC184          | Cm′ Te′                   | Laboratory collection |
| pCY314            | Cm′ acpS under araBAD promoter in pACYC184 | Laboratory collection |
| pYON108           | acpS1 allele from MP4 under araBAD promoter in pACYC184 | This work |
| pYON110           | Cm′ Te′ derivative of pACYC184 | This work |
| pYON113           | yhhU under araBAD promoter in pBAD22 | This work |
| pDLC140           | rnc and pdsJ operons inserted into pBR322 | 10 |
| pTX290            | Ap′                       | 11 |
| pK18              | Kan′                      | 18 |
| pRL101            | Ap′ acpS under T7 promoter in pET22b | 12 |
| pRL102            | Ap′ acpS under T7 promoter in pET22b | This work |

* Antibiotic resistance to ampicillin, chloramphenicol, kanamycin, and tetracycline is indicated by Ap′, Cm′, Kan′, and Te′, respectively. Te′ indicates sensitivity to tetracycline.

Subsequent identification of dpj as the gene encoding the E. coli AcpS activity resulted in the renaming of dpj to acpS (12). This was the first gene known to encode a phosphopantetheinytransferase, and its discovery renewed general interest in PPTases and the role of the 4′-PP post-translational modification in activating the synthases that produce a myriad of secondary metabolites (13). The E. coli acpS sequence led to the identification and subsequent confirmation of other PPTases in a variety of organisms (14). This includes two additional PPTases in E. coli: EntD, which phosphopantetheinylates the EntB and EntF members of the Ent biosynthetic gene cluster responsible for producing the enterobacterin siderophore (15), and YhhU, a previously uncharacterized open reading frame of 195 amino acids (called o195) (14). Both the entD and yhhU gene products were demonstrated to modify apoACP in vitro at a very low rate (14). EntD has been shown to efficiently modify EntF in vitro, which validates an in vivo role of EntD in activating EntF consistent with the co-induction of the two genes and their presence in the same operon. The very low in vitro activity of the YhhU protein in modifying EntF and ACP led to the conclusion that the physiological substrate was an undiscovered acceptor protein (14).

### Identification of the Mutation in the acpS Gene of E. coli MP4—

In the Boston laboratory, four independent PCR amplifications of the acpS gene from the E. coli MP4 strain were performed using the forward primer 5′-CCGACATCCCTGAGATGCATGA-3′ and the reverse primer 5′-ACACTGATCCATAATCGCTGT-3′. These primers anneal to the E. coli chromosome at approximately 100 bases upstream and downstream from the acpS start and stop codons, respectively. PCR products were purified by agarose gel electrophoresis, and sequencing revealed a mutation in the acpS of the E. coli MP4 strain (denoted acpS1) containing a single G to A transition in the fifth codon (underlined), 5′-ATG-GCAATATTAGAT-3′. This mutation corresponds to a G4D substitution in the expressed protein (denoted AcpS1). This mutation was confirmed in the Urbana laboratory upon sequencing of the PCR product obtained using another set of primers.

### Cloning of acpS from E. coli MP4 for In Vitro Kinetic Studies—

acpS1 was PCR-amplified using a forward primer that incorporated an NdeI restriction site (underlined) and the start codon 5′-TGACCTCAGCATATGCCATAGA-3′. The reverse primer incorporated a HindIII restriction site (underlined) after the stop codon 5′-ATGACCTCAGCATATGCCATAGA-3′. The resulting PCR product was digested with NdeI and HindIII, then subcloned into the corresponding restriction sites in the pET22 expression plasmid from Novagen, Inc. This construct was designated pRL102 and subsequently transformed into BL21(DE3), creating the strain RL102 for overexpression of the AcpS1 protein.
Cloning of acpS for in Vivo Complementation Studies—The acpS plasmid pCY314 was made by ligating the SphI-HindIII yhhU fragment of pTX290 (11) to the annealed product of two phosphorylated oligonucleotides, 5′-GGCCTGGCGGCATGTCGCTAATGAGCTTGATGATCAGTTAACTGAATCG-3′ and 5′-GCATGCTGCACAAAATTAAAG-3′. The ligated product was then ligated to pK18 (18) digested with Aec65I and HindIII. The resulting gene was then amplified by AflIII digestion (underlined) followed by melting the acpS initiation codon. The Ncol-HindIII fragment that encodes a wild type acpS protein was then ligated to NcoI plus the HindIII digest of pBAD22. Finally the Cla1-ScaI fragment of this construct was replaced with the Cla1-HindII fragment of pACYC184 to give pCY314.

Cloning of acpS1 from E. coli MP4 for in Vivo Complementation Studies—A PCR-amplified product from the E. coli MP4 strain was amplified and cloned into the BamH I site in the following manner. An NcoI site (underlined) was engineered at the start codon using primers 5′-GGTGGTCCCATGGCAATAT-3′ and 5′-GCACTGTCGCAACAAAAATGAGG-3′. The PCR product was blunt-ended with mung bean nuclease from New England Biolabs, Inc. and subsequently digested with NcoI. The NcoI/blastu fragment was ligated into Ncol/SmaI double-digested pBAD22 (19) to produce pYON107. To reduce the copy number, the ClaI/NotI site of this construct was blunt-ended with mung bean nuclease from New England Biolabs, Inc. and subsequently digested with ClaI. The ClaI/NotI digest was ligated into NotI double-digested pBAD22 (19) to produce pYON107. To reduce the copy number, the ClaI/NotI digest was ligated into Ncol/HindIII and ligated into pBAD22 digested with Ncol and HindIII.

Purification of Holo-(Acyl Carrier Protein) Synthases—AcpS was overexpressed from strain RIL101 and purified according to a previously developed method (12). An additional purification step for the protein was appended to this method employing Sephacryl S-300 size exclusion chromatography from Amersham Pharmacia Biotech to remove a high molecular weight contaminant. AcpS1 from the wild type strain W3110 using an amino-terminal primer containing an introduced AflIII site (underlined) 5′-GGGCCGCATGATGATCAGTTAACTGAATCG-3′ and a carboxyl-terminal primer containing an introduced HindIII site (underlined) 5′-GGCGGTTAAGCTTATCAGTTAACTGAATCG-3′. The PCR products were digested with AflIII plus HindIII and ligated into pBAD22 digested with NcoI and HindIII.

Purification of Apocarboxylic Carrier Proteins—The E. coli strains DK554, DK676, and DK700 were used to overexpress the wild type, S36A, and S36T E. coli ACPs, respectively (20). Purification of apoACP was performed using a scheme involving a freeze/thaw osmotic shock release of ACP from cells followed by anion exchange chromatography with Q Sepharose from Amersham Pharmacia Biotech to remove a high molecular weight contaminant. AcpS1 from the E. coli MP4 strain was overexpressed from strain RIL102. Purification of the AcpS1 was performed in a manner identical to wild type AcpS. Amino-terminal protein sequencing of the purified acpS1 confirmed the presence of the G4D mutation.

Analysis of in Vivo ACP Pools—ApoACP was analyzed by urea-PAGE as described previously (23) with the following modifications. Overnight cultures were diluted into fresh medium subcultured several generations to mid-log phase and then treated with 5% trichloroacetic acid. After centrifugation, the protein pellets were washed twice (at 4 °C) with 1% trichloroacetic acid and resuspended in 1 mM urea. These samples were then subjected to a 13% PAGE with 1 mM urea. The separated proteins were subsequently visualized by staining with Coomassie Blue.

RESULTS

Kinetic Characterization of AcpS—Since the acpS1 mutation was suppressible by high intracellular CoA levels, the phenotype of the E. coli MP4 mutant strain seemed likely to be explained by kinetics of the mutant enzyme. However, the kinetic parameters of the wild type enzyme had to first be determined. Studies of the 4′-PP post-translational modification catalyzed by the wild type AcpS were performed with the 4′-3H]PP transfer assay. This assay allows for direct measurement of apoACP to holoACP conversion via trichloroacetic acid precipitation of the 3H-labeled holo protein and subsequent quantification by liquid scintillation counting. The predominant kinetic feature of AcpS was the severe substrate inhibition of the enzyme by apoACP at concentrations >5 μM (Fig. 1, panel A). AcpS had a Kₘ of 1.5 μM for apoACP and a peak 4′-PP transfer activity of k_cat of 65 min⁻¹, occurring at an apoACP concentration of 5 μM. The kinetic profile of AcpS as a function of apoACP concentration and all kinetic data displaying substrate inhibition have been fit by linear regression with the general substrate inhibition equation of Cleland (24). AcpS exhibits Michaelis-Menten kinetics and a Kₘ of 50 μM with respect to CoA as the variable substrate (Fig. 1, panel B).

NaCl Effects on AcpS Kinetics—One explanation for the observed substrate inhibition was an electrostatic interaction between the basic AcpS enzyme (pI 9.6) and the acidic apoACP substrate (pI 4.1), which should be suppressed by high salt concentrations. Indeed, at an NaCl concentration of 250 mM, a moderate relaxation in the substrate inhibition of AcpS by apoACP was observed (Fig. 2). This relaxation was characterized by a slight increase in the Kₘ for apoACP and an increase in the k_cat of the enzyme at saturating apoACP concentration. The maximum observed activity for AcpS in the presence of 250 mM NaCl is 62 min⁻¹. At 500 and 750 mM NaCl, substrate inhibition of AcpS was dramatically reduced, and the enzyme exhibited near Michaelis-Menten behavior (Fig. 2). The enzyme continued to maintain a peak activity of ~92 min⁻¹. The apoACP concentration at which peak activity occurred increased ~10-fold as a function of the NaCl concentration, from 4 to 40 μM.

Inhibitors of AcpS—Prior in vitro work (20) showed that a mutant ACP in which the target serine 36 had been mutated to a threonine residue was an inactive substrate for phosphopantetheinylation. This protein was tested to determine if it was an inhibitor of AcpS as well as an inactive substrate. The in vitro results were first confirmed in vitro. S36T apoACP was...
overexpressed and purified to >99% homogeneity. In addition, S36A apoACP was prepared in an identical manner as a non-phosphopantetheinylatable control. Incubations of AcpS with [3H]CoA and either apoACP, S36A apoACP, or S36T apoACP were conducted for periods up to 5 h. The results of these assays clearly indicated the absence of any 4'-[3H]PP labeling of S36T apoACP or the S36A apoACP control, although the wild type ACP was readily labeled. The apparent inability of AcpS to phosphopantetheinylate S36T apoACP was also confirmed through separation of the apo and holo forms of ACPs after 1-h incubations with AcpS and CoA, using a previously developed HPLC method (21, 22).

S36A and S36T apoACPs were then characterized as inhibitors of AcpS using the 4'-[3H]PP transfer assay. Several incubations of AcpS with 10 μM apoACP were performed with successively increased concentrations of either S36A apoACP or S36T apoACP in the absence of NaCl as well as in the presence of 500 mM NaCl (Fig. 3). An IC50 of 10 μM was obtained for S36A apoACP (Fig. 3, panel A). This value remained nearly the same, regardless of the presence of NaCl in the reaction mixtures. An IC50 of 7 μM was obtained for S36T apoACP in the presence of 500 mM NaCl (Fig. 3, panel B). An IC50 value for S36T apoACP in reactions conducted in the absence of NaCl could not be obtained, because it was observed that adding successively greater concentrations of S36T apoACP to these incubations resulted in a slight increase in AcpS activity.

Ability of S36T apoACP to Relieve AcpS Substrate Inhibition—in response to the observation that successively increasing amounts of S36T apoACP increased AcpS catalysis of 4'-PP transfer to apoACP in the absence of NaCl, a kinetic study of the S36T apoACP activation of AcpS was performed. 100 μM S36T apoACP was added to a series of 4'-[3H]PP transfer assays investigating AcpS catalytic activity with respect to the apoACP substrate concentration (Fig. 4). Comparison of the results of this study with the previous characterization of AcpS kinetics performed in the absence of S36T apoACP clearly indicated that S36T apoACP activates AcpS through relief of substrate inhibition by apoACP. In the presence of 100 μM S36T apoACP, AcpS had Michaelis-Menten kinetics with a kcat of 80 min−1 and a Km of 18 μM for apoACP.

Identification of G4D Mutation in AcpS1—The E. coli MP4 phenotype had been attributed to a deficiency of AcpS activity due to expression of a mutant allele (denoted acpS1) encoding a protein having a postulated elevated Km for CoA (9). PCR amplification and subsequent DNA sequencing of acpS1 from E. coli MP4 revealed that the fifth codon contained a single G to A transition. This base change resulted in a glycine to aspartate substitution at the fourth residue in the final acpS1 gene product. Recombinant E. coli acpS1 was expressed and
purified in a manner identical to that used for AcpS. The G4D mutation in the purified AcpS1 protein was confirmed by amino-terminal protein sequencing.

Kinetic Characterization of AcpS1—Kinetic characterization of AcpS1 was conducted in a manner analogous to that of AcpS. The AcpS1 enzyme showed a 5-fold reduction in its catalytic activity as a result of the G4D mutation, with a $k_{cat}$ of 7.5 min$^{-1}$ at saturating apoACP concentration and a peak activity of $k_{cat} = 10$ min$^{-1}$ at an apoACP concentration of 5 $\mu$M (Fig. 1, panel A). Substrate inhibition of AcpS1 by apoACP was significantly less severe than that which was observed for AcpS. The AcpS1 $K_m$ for apoACP was 2.5 $\mu$M, only marginally greater than that observed for AcpS. AcpS1 had Michaelis-Menten kinetics with respect to the CoA substrate with a $K_m$ of 75 $\mu$M (Fig. 1, panel B).

acpS Mutants Accumulate ApoACP in Vivo—Previous studies of acpS activity in vivo utilized the E. coli MP4 acpS1 mutant strain, which has a complex phenotype (9). Since a characterization of the AcpS1 protein in vivo was desired, the more straightforward mutant E. coli HT253 strain of Takiff et al. (10) seemed ideal because under nonpermissive conditions this strain should produce no AcpS protein. This lack of protein production would preclude formation of mixed AcpS dimers (having a mutant subunit and a wild type subunit) which could complicate the phenotypic analysis. However, since strain HT253 had not been examined biochemically, the ACP species present in vivo were examined under both the permissive and nonpermissive conditions. It was expected that if AcpS is the primary or only enzyme capable of carrying out the 4'-PP modification of ACP in E. coli, then a mutation that affects the transcription of acpS should hinder modification of apoACP and result in considerable accumulation of intracellular apoACP. The acpS tetracycline-dependent, conditional mutant E. coli HT253 strain was grown overnight under permissive conditions (the presence of tetracycline to induce the tet promoter), then subcultured under either permissive or nonpermissive conditions. The ACP species present in vivo were examined under both the permissive and nonpermissive conditions. It was expected that if AcpS is the primary or only enzyme capable of carrying out the 4'-PP modification of ACP in E. coli, then a mutation that affects the transcription of acpS should hinder modification of apoACP and result in considerable accumulation of intracellular apoACP. The acpS tetracycline-dependent, conditional mutant E. coli HT253 strain was grown overnight under permissive conditions (the presence of tetracycline to induce the tet promoter), then subcultured under either permissive or nonpermissive (absence of tetracycline) conditions. The ACP species present in vivo were examined under both the permissive and nonpermissive conditions. It was expected that if AcpS is the primary or only enzyme capable of carrying out the 4'-PP modification of ACP in E. coli, then a mutation that affects the transcription of acpS should hinder modification of apoACP and result in considerable accumulation of intracellular apoACP. The acpS tetracycline-dependent, conditional mutant E. coli HT253 strain was grown overnight under permissive conditions (the presence of tetracycline to induce the tet promoter), then subcultured under either permissive or nonpermissive (absence of tetracycline) conditions. The ACP species present in vivo were examined under both the permissive and nonpermissive conditions. It was expected that if AcpS is the primary or only enzyme capable of carrying out the 4'-PP modification of ACP in E. coli, then a mutation that affects the transcription of acpS should hinder modification of apoACP and result in considerable accumulation of intracellular apoACP.
The acpS1 Allele of MP4 Is Responsible for the Mutant Phenotype—Experiments were undertaken to demonstrate that the acpS1 of the E. coli MP4 strain is responsible for its mutant phenotype, where high intracellular CoA concentrations are required for growth. The acpS1 gene of the E. coli MP4 strain was cloned behind an inducible araBAD promoter, and the ability to complement the tetracycline-dependent acpS conditional mutant E. coli HT253 strain was tested (Fig. 6). Unlike the wild type acpS plasmid pCY314, which complemented the mutant without induction of the arabinose promoter, the plasmid pYON108 carrying the acpS1 mutant allele complemented the mutation only upon induction by addition of 0.4% arabinose (Fig. 6, plate 3). This experiment indicated that the acpS1 allele encoded a less active enzyme and that it is this mutant gene which is responsible for the phenotype of the E. coli MP4 strain, requiring elevated CoA levels for growth.

Additional studies of acpS1 examined phenotypes of strains using the procedure of Polacco and Cronan (9), which was originally employed in the identification of the E. coli MP4 strain. This assay examined the growth of strains carrying a panB6 lesion in minimal media supplemented with pantothenate in order to distinguish among strains requiring different levels of intracellular CoA for viability.

Strain RF103 was generated by transforming the E. coli MP4 strain with the complementing plasmid pDLC140, which carries the wild type acpS under the control of its native promoter. Growth of this strain in minimal media supplemented with 0.25 mM pantothenate and in the presence of ampicillin was monitored by light absorbance at wavelength 600 nm. Comparison of the growth phenotype of the strain RF103 with strains RF101 (MP3 carrying pBR322) and RF102 (MP4 carrying pBR322) indicates a partial recovery of the mutant phenotype through direct complementation of acpS1 (Fig. 7, panel A).

Strain RF104 was generated through P1 transduction, whereby the chromosomal acpS1 of the E. coli MP4 strain was
replaced with wild type acpS. Phenotyping of this strain using the Polaco and Cronan (9) assay also revealed partial recovery of the MP4 phenotype when compared with strains MP3 and MP4 (Fig. 7, panel B).

Overproduction of YhhU Suppressed the acpS Mutation of HT253—The exact role of the third E. coli PPTase YhhU remains unclear (14). As with EntD, studies revealed that YhhU modified apoACP in vitro with very low efficiency (14). However, there were two caveats to these findings. First, the protein was produced as inclusion bodies, and refolding to the native structure was assumed but not demonstrated. Second, the protein produced contained an extra glycine residue adjacent to the initiation methionine. Since the mutation in acpS1 of the E. coli MP4 strain indicated that the amino terminus played an important role in enzyme activity, this extra residue might have compromised the activity of the protein.

The ability of the YhhU product to modify apoACP in vivo was tested. The yhhU gene was placed behind the araBAD promoter to give pYON113, in which the gene was expressed from the plasmid promoter and ribosome binding site. When transformed into the tetracycline conditional E. coli HT253 strain and induced with 0.4% arabinose, YhhU fully complemented the tetracycline-dependent growth phenotype of the strain (Fig. 8, plate 3) and blocked the accumulation of apoACP (Fig. 9, lane 4). However, when expression off the arabinose promoter was not induced or was decreased by the addition of glucose to the medium, growth of the strain was tetracycline-dependent (Fig. 8, plate 2 and Fig. 9, lane 3). These results confirm the ability of the yhhU gene product to functionally replace AcpS in vivo but only when expressed at high levels. It is proposed that this gene be named acpT to reflect this property.

**DISCUSSION**

Phosphopantetheinyl transfer by AcpS was studied in vitro, establishing a context for understanding the physiology of holo-ACP formation in E. coli. The predominant feature of AcpS steady state kinetics is the unusually severe substrate inhibition of the enzyme by apoACP (Fig. 1). Although this effect crippled AcpS catalytic activity by nearly 50% at apoACP concentrations above 10 μM, there appeared to be no physiological basis for the accumulation of apoACP and substrate inhibition of the enzyme in vivo. Previous studies of in vivo ACP pools reveal that the apo form of the protein is not detectable in wild type E. coli (25), implying a rapid and efficient conversion of the protein to the holo form by AcpS. Indeed, the overexpression and accumulation of apoACP in E. coli has been shown to be toxic (20).

Factors that relieve AcpS substrate inhibition in vitro were identified, providing clues about a possible biophysical mechanism(s) responsible for this effect. High concentrations of NaCl in the 250 to 750 mM range elicited a dramatic relief of the substrate inhibition of AcpS by apoACP (Fig. 2). This effect is attributed specifically to the Na⁺ cation rather than the Cl⁻ anion, since other monovalent and divalent chloride salts have different impacts on AcpS kinetics.² Monovalent and divalent cations are known to activate specific component enzymes in E. coli fatty acid biosynthesis (26) and can influence ACP conformational stability and structure (27). It should be noted that the severe substrate inhibition of AcpS observed in the absence of NaCl is not likely to represent the true intracellular kinetic profile of the enzyme, given the ionic content of cells.

The presence of S36T apoACP was also shown to relieve substrate inhibition of AcpS by apoACP in the absence of NaCl (Fig. 4), even though S36T apoACP itself cannot be phosphopantetheinylated.² The introduction of the γ-CH₂ in S36T apoACP blocked the conversion of this protein to the holo form, but the binding of S36T apoACP by AcpS may induce an allostERIC change in the homodimeric enzyme that relieves substrate inhibition by apoACP. Based on these findings, it is believed that AcpS substrate inhibition may arise from the presence of multiple apoACP conformers, improper binding of apoACP to active site(s) on AcpS, and/or negative cooperativity between apoACP binding sites on the AcpS homodimer. Additional structural information about AcpS will be required to fully understand the nature of this complex kinetic behavior.

Having obtained a reasonable kinetic profile for phosphopantetheinyl transfer by AcpS, the physiology of the E. coli MP4 strain and the basis for its deficiency in holoACP synthesis were investigated. The E. coli MP4 strain was phenotypically selected as a strain requiring growth medium supple-
mented with an unusually high 25 μM pantothenate concentration (a CoA precursor) for viability. At 0.25 μM pantothenate, a level sufficient to support growth of strains having a wild type acpS gene, E. coli MP4 cultures ceased growth and had barely detectable holoACP levels. When supplemented with 25 μM pantothenate, E. coli MP4 cultures grew and contained a mixture of both apo and holo forms of ACP. The rationale of this selection was that the addition of high levels of pantothenate to the growth medium would increase the intracellular CoA concentration and allow function of a mutant AcpS activity having an elevated $K_m$ for CoA (9).

Sequencing of the acpS gene from the E. coli MP4 strain (denoted acpS1) identified a G4D mutation located immediately amino-terminal to the (V/I)G(V/I)D motif conserved among currently known PPTases (14). The kinetic profile of the AcpS1 protein in vitro did not reveal a significantly elevated $K_m$ for CoA. Instead, it is believed to be the 5-fold reduction in catalytic efficiency $k_{cat}/K_m$ of AcpS1 that results in phenotypic 2- to 10-fold lower in vivo holoACP content of the E. coli MP4 strain.

Although it had been shown that a point mutation existed in the acpS1 gene isolated from the E. coli MP4 strain, the possibility that a second mutation elsewhere in the chromosome might be responsible for the elevated CoA growth phenotype of this mutant had not been ruled out. Therefore, genetic analysis of the acpS1 allele of the E. coli MP4 strain was undertaken. Complementation of the tetracycline-dependent acpS strain HT253 by overproducing the acpS1 allele carrying the MP4 point mutation was successful (Fig. 6). In addition, complementation and chromosomal replacement of the acpS1 with wild type acpS in strains RF103 and RF104, respectively, resulted in partial recovery of the MP4 growth phenotype (Fig. 7). These results suggest that the mutation in the acpS1 gene is largely responsible for the high in vivo CoA concentrations required by the E. coli MP4 strain for growth. The original mapping of the

![Fig. 8. Suppression of the tetracycline-dependent acpS conditional strain HT253 by yhhU expressed from the araBAD promoter.](image)

Plate 1, medium containing tetracycline. Plate 2, medium lacking tetracycline. Plate 3, medium lacking tetracycline but supplemented with 0.4% arabinose for induction of gene expression. Section A, strain containing pBAD22 (vector). Section B, strain carrying pYON113 (yhhU).

![Fig. 9. Modification of apoACP in strain HT253 upon overexpression of yhhU.](image)

13% urea-PAGE of proteins stained with Coomassie Blue. Lane 1, strain containing pBAD22 (vector) in medium containing tetracycline and glucose. Lane 2, strain containing pBAD22 (vector) medium containing tetracycline and arabinose. Lane 3, strain containing pYON113 (yhhU) with medium containing tetracycline and glucose. Lane 4, same as lane 3 except that the medium contained arabinose and lacked tetracycline. Lane 5, strain containing pYON113 in medium containing tetracycline and arabinose. Lane 6, apoACP standard.
There is evidence that the two other *E. coli* PPTases, EntD and YhhU, are capable of inefficiently modifying apoACP in vitro (14). Therefore, the ability of EntD and YhhU to modify apoACP was tested in vivo. Expression of EntD and other genes involved in enterobactin biosynthesis is under the regulation of Fur, a transcriptional regulator that responds to intracellular iron concentrations (29). The presence of intracellular iron is sensed by Fur, and Fur correspondingly acts as a transcriptional repressor for iron-regulated genes. Regulation of AcpS has not been observed to be repressed by Fur, and it is unlikely that residual expression of EntD under normal, iron-rich conditions would provide sufficient activity to modify apoACP in an *acps*− strain. Nonetheless, *acps* suppression by derepression of *entD* by iron starvation was examined, and no suppression of *acps* was found under these conditions. Cloning of *entD* under an inducible promoter and testing its ability to suppress an *acps*− phenotype gave a negative result, despite the fact that the EntD that was expressed was capable of phosphopantetheinylating EntF in crude cell extracts. This provides further evidence that *entD* is not able to suppress *acps* and modify apoACP in vivo.

Up to this time, not much was known about YhhU other than the fact that it has sequence homology with other PPTases. Similar to *entD* above, the *yhhU* gene was cloned under an inducible promoter to test for the ability to suppress an *acps*− phenotype. Induction of *yhhU* allowed the HT253 conditional strain to grow in nonpermissive media lacking tetracycline, as well as completely modifying all of the apoACP pool (Figs. 8 and 9). The fact that the *in vitro* analysis of YhhU demonstrated such low activity on apoACP (14) does not necessarily contradict this result. One of the problems with the *in vitro* analysis of YhhU was that the initial attempts in cloning and purification of YhhU led to an additional glycerine after the start site. Another problem was that the purification process led to an inclusion-body form of YhhU that might have not been properly refolded (14). Improper folding of YhhU could have been responsible for the low *in vitro* activity. This work demonstrated that YhhU is able to modify apoACP when overexpressed in conjunction with an *acps* mutation.

One possible explanation of *acps* suppression by excess YhhU is that *E. coli* evolved two enzymes that are capable of modifying apoACP due to its crucial role in cell survival. In a normal cell, AcpS could be the primary enzyme with more efficient activity. The secondary enzyme, YhhU, is less active and thus plays a major role in 4′-PP modification of apoACP. Mutations in *acps* could possibly allow selection for higher expression of the secondary enzyme to provide apoACP modification for survival.

Previous physiological studies originally revealed the ability of the *E. coli* AcpS to maintain complete holoACP pools *in vivo* under normal conditions. The results presented in this work indicate that AcpS also exists at levels minimally required and operates with an efficiency minimally necessary to ensure the complete conversion of apoACP to holoACP. A disruption of AcpS production or a small reduction of AcpS catalytic efficiency are capable of reducing phosphopantetheinyl transfer to the point where significant (and possibly toxic) levels of apoACP accumulate *in vivo*. Under circumstances where AcpS activity is compromised, possible mechanisms for physiological recovery by *E. coli* have been identified and involve elevations in intracellular CoA pools or increased expression of the complementing PPTase YhhU such that *in vivo* holoACP is maintained at levels sufficient for cellular viability.

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3 Y. Hwangbo, unpublished results.
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